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(54) Title: PROTEIN SEPARATION AND DISPLAY

(57) Abstract: The present invention relates to multi-phase protein separation methods capable of resolving and characterizing large numbers of cellular proteins, including methods for efficiently facilitating the transfer of protein samples between separation phases. In particular, the present invention provides an automated system for the separation, identification, and characterization of protein samples. The present invention thus provides improved emthods for the analysis of samples containing large numbers of proteins.

PROTEIN SEPARATION AND DISPLAY

This application claims priority benefit of U.S. Provisional Appln. Ser. Nos. 60/180,911, filed 02/08/00, 60/239,325, filed 10/10/00, 60/239,326, filed 10/10/00, and 60/259,448, filed 01/03/01, each of which is herein incorporated by reference in their

5   entireties.

10   ## FIELD OF THE INVENTION

The present invention relates to multi-phase protein separation methods capable of resolving and characterizing large numbers of cellular proteins, including methods for efficiently facilitating the transfer of protein samples between separation phases. In particular, the present invention provides an automated system for the separation,

15   identification, and characterization of protein samples.

BACKGROUND OF THE INVENTION

As the nucleic acid sequences of a number of genomes, including the human genome, become available, there is an increasing need to interpret this wealth of information. While the availability of nucleic acid sequence allows for the prediction

20   and identification of genes, it does not explain the expression patterns of the proteins produced from these genes. The genome does not describe the dynamic processes on the protein level. For example, the identity of genes and the level of gene expression does not represent the amount of active protein in a cell nor does the gene sequence describe post-translational modifications that are essential for the function and activity

25   of proteins. Thus, in parallel with the genome projects there has begun an attempt to understand the proteome (*i.e.*, the quantitative protein expression pattern of a genome under defined conditions) of various cells, tissues, and species. Proteome research

seeks to identify targets for drug discovery and development and provide information for diagnostics (*e.g.*, tumor markers).

An important area of research is the study of the protein content of cells (*i.e.*, the identity of and amount of expressed proteins in a cell). This field requires

5   methods that can separate out large numbers of proteins and can do so quantitatively so that changes in expression or structure of proteins can be detected. The method generally used to achieve such cellular protein separations is 2-D PAGE. This method is capable of resolving hundreds of proteins based upon pI in one dimension and protein size in the second dimension. The proteins separated by this method are

10   visualized using a staining method that can generally be quantified. The result is a 2-dimensional image where the protein map is based on pI and approximate molecular weight. By the use of computer based image analysis techniques, one can search for proteins that are differentially expressed in various cell lines. These methods are used to monitor changes in protein expression that are linked to conditions such as cell

15   transformation and cancer progression, cell aging, the response of cells to environmental insult, and the response of cells to pharmaceutical agents. Once changes in protein expression have been identified, then one can further analyze target proteins to determine their identity and whether they have been altered from their expected structure by sequence changes or post-translational modifications.

20   Although 2-D PAGE is still widely used for protein analysis, the method has several limitations including the fact that it is labor intensive, time consuming, difficult to automate and often not readily reproducible. In addition, quantitation, especially in differential expression experiments, is often difficult and limited in dynamic range. Also, while the 2-D gel does produce an image of the proteins in the cell, the mass

25   determination is often only accurate to 5-10%, and the method is difficult to interface to mass spectrometric techniques for further analysis.

Another limitation of 2-D PAGE is the amount of protein loaded per gel which is generally below 250 µg. The amount of protein in any given spot may therefore be too low for further analysis. For Coomassie brilliant blue (CBB) stained gels the limit

30   of detection is 100 ng per spot while for silver stained gels the limit of detection is 1 –

10 ng. Furthermore, proteins that have been isolated in 2-D gels are embedded inside the gel structure and are not free in solution, thus making it difficult to extract the protein for further analysis. Because of these limitations, the art is in need of protein mapping methods that are efficient, automated, and have broader resolution capabilities than presently available technologies.

SUMMARY OF THE INVENTION

The present invention relates to multi-phase protein separation methods capable of resolving and characterizing large numbers of cellular proteins, including methods for efficiently facilitating the transfer of protein samples between separation phases. In particular, the present invention provides an automated system for the separation, identification, and characterization of protein samples.

For example, the present invention provides a method for displaying proteins comprising: 1) providing i) a sample comprising a plurality of proteins, ii) a first separating apparatus, and iii) a second separating apparatus; 2) treating the sample with the first separating apparatus to produce a first separated protein sample, wherein the first separated protein sample is collected from the first separating apparatus in a plurality of fractions, each of the fractions defined by a distinct pH range (*i.e.*, each of the fractions containing proteins that are eluted from the first separating apparatus within a particular pH range); treating the first separated protein sample with the second separating apparatus to produce a second separated protein sample; and analyzing the second separated protein sample by mass spectrometry to produce a 2-dimensional protein map. In preferred embodiments, the 2-dimensional protein map comprises a first dimension representing protein pI point. In yet other preferred embodiments, the 2-dimensional protein map further comprises a second dimension representing protein molecular weight.

The present invention also provides a system comprising: a first separating apparatus; a buffer capable of eluting protein from the first separating apparatus in a plurality of fractions; a first delivery apparatus capable of receiving separated protein in the plurality of fractions, each of the fractions defined by a distinct pH range; a

second separating apparatus, wherein the second separating apparatus is configured to

receive proteins from the first delivery apparatus; a second delivery apparatus capable

of receiving separated protein from the second separating apparatus; a mass

spectrometry apparatus configured to receive proteins from the second delivery

5   apparatus; and a processor connected to the system, wherein the processor is capable

of producing a 2-dimensional data representation of separated proteins analyzed by the

mass spectrometry apparatus. In preferred embodiments, the system further comprises

a display system capable of displaying the data representation. In other preferred

embodiments, the 2-dimensional data representation comprises a first dimension

10   representing protein pI point, while in yet other preferred embodiments, the 2-

dimensional data representation further comprises a second dimension representing

protein molecular weight.

        The present invention further provides a method for displaying proteins

comprising providing: i) a sample comprising a plurality of proteins, ii) an isoelectric

15   separating apparatus, and iii) a reverse phase HPLC separating apparatus; treating the

sample with the isoelectric separating apparatus to produce a first separated protein

sample; treating said first separated protein sample with said reverse phase HPLC

separating apparatus to produce a second separated protein sample; and analyzing the

second separated protein sample with mass spectrometry (e.g., ESI oa TOF/MS) to

20   produce a 2-dimensional protein map. In preferred embodiments, the isoelectric

separating apparatus comprises a liquid phase separating apparatus. In yet further

preferred embodiments, the reverse phase HPLC comprises non-porous reverse phase

HPLC. In some preferred embodiments, the method further comprises the step of

determining the identity of at least one protein in the 2-dimensional protein map. For

25   example, in some embodiments, the identifying comprises comparing at least a portion

of said 2-dimensional protein map with a control protein map.

        In some preferred embodiments, the sample comprising the plurality of proteins

further comprises a buffer, wherein the plurality of proteins are solubilized in the

buffer and wherein the buffer is compatible with the two separating apparatuses. In

yet other preferred embodiments, the buffer is further compatible with ESI oa TOF/MS.

The present invention also provides a system comprising: an isoelectric separating apparatus; a first delivery apparatus capable of receiving separated protein
5   from the isoelectric separating apparatus; a reverse phase HPLC separating apparatus, wherein the HPLC separating apparatus is configured to receive proteins from the first delivery apparatus; a second delivery apparatus capable of receiving separated protein from the reverse phase HPLC separating apparatus; and an ESI oa TOF/MS apparatus configured to receive proteins from the second delivery apparatus. In some
10   embodiments, the system further comprises a processor connected to the system, wherein the processor is capable of producing a data representation of separated proteins analyzed by the ESI oa TOF/MS apparatus. In yet other embodiments, the system further comprises a display system capable of displaying the data representation. In preferred embodiments, the display system is capable of displaying
15   the data representation as a 2-dimensional protein map. In particularly preferred embodiments, the 2-dimensional protein map displays a mass spectra in a top view protein band format. In other preferred embodiments, the 2-dimensional protein map has a resolution that allows for the differentiation between phosphorylation states of a protein or other minor variations (*e.g.*, difference in methylation, glycosylation,
20   lipidation, truncation, amino acid substitutions, etc.). In preferred embodiments, the isoelectric separating apparatus comprises a liquid phase separating apparatus. In yet other preferred embodiments, the reverse phase HPLC apparatus comprises a non-porous reverse phase HPLC apparatus.

The present invention further provides a method for displaying proteins
25   comprising providing: a sample comprising a plurality of proteins, a first separating apparatus, wherein the first separating apparatus is capable of (*i.e.*, is configured for) separating proteins based on a first physical property, and a second separating apparatus, wherein the second separating apparatus is a liquid phase separating apparatus and wherein the second separating apparatus is capable of (*i.e.*, is configured
30   for) separating proteins based on a second physical property; treating the sample with

the first separating apparatus to produce a first separated protein sample; treating the first separated protein sample with the second separating apparatus to produce a second separated protein sample; and displaying at least a portion of the second separated protein sample under conditions such that the first and the second physical properties

5   of at least a portion of the plurality of proteins are revealed. In some preferred embodiments, the first and the second physical properties include, but are not limited to, charge, hydrophobicity, and molecular weight. In some embodiments, the displaying results in a two-dimensional display while in other embodiments the display is three-dimensional (e.g., display with a third physical property) or multi-dimensional.

10   In some embodiments, the sample comprising a plurality of proteins further comprises a buffer, wherein said plurality of proteins are solubilized in the buffer and wherein the buffer is compatible with the first and said second separating apparatus. In some preferred embodiments, the buffer is further compatible with mass spectrometry. In some embodiments, the buffer comprises a compound of the formula

15   n-octyl SUGARpyranoside (e.g., n-octyl C_6-C_{12} glycopyranoside, where C_6-C_{12} glycopyranoside is a six to twelve carbon sugar pyranoside). The sugar component is not limited to any particular sugar and includes compounds such as n-octyl ß-D-glucopyranoside and n-octyl ß-D-galactopyranoside.

       In some preferred embodiments, the sample comprises a cell lysate (e.g., cells

20   from animals, plants, and microorganisms; cancer cells; tissue culture cell; cells at various stages of development or differentiation; embryonic cells; tissues; etc.). However, the present invention is not limited to the use of cell lysates. For example, the sample may comprise purified and partially purified protein preparations. The present invention is also not limited in the nature of the proteins. For example,

25   proteins may include, but are not limited to, protein fragments, polypeptides, modified proteins (e.g., lipidated, glycosylated, phosphorylated etc.), protein complexes (e.g., protein/protein, protein/nucleic acid), acid proteins, basic proteins, hydrophobic proteins, hydrophilic proteins, membrane proteins, cell surface proteins, nuclear proteins, transcription factors, structural proteins, enzymes, receptors, and the like.

In some embodiments of the present invention the first separating apparatus comprises a liquid phase separating apparatus. However, the first separating apparatus is not limited to liquid phase apparatuses. For example, the first separating apparatus may be gel-based or may be selected from methods including, but not limited to, ion

5   exclusion, ion exchange, normal/reversed phase partition, size exclusion, ligand exchange, liquid/gel phase isoelectric focusing, and adsorption chromatography. In some preferred embodiments, the first separating apparatus comprises an isoelectric focusing apparatus. In some embodiments of the present invention, the second separating apparatus comprises reverse phase HPLC. In some preferred embodiments,

10   the reverse phase HPLC comprises non-porous reverse phase HPLC. Certain embodiments of the present invention may utilize a second separation apparatus that is not liquid phase (e.g., gel-phase).

In some embodiments of the present invention the method further comprises the step of determining the identify of at least one protein of the second separated protein

15   sample. Although the present invention is not limited to any particular method of determining the identify of the protein, in some embodiments, the method comprises analyzing said at least one protein from the second separated protein sample with mass spectrometry.

The present invention also provides a method for characterizing proteins

20   comprising providing: i) a sample comprising a plurality of proteins, ii) a first separating apparatus, wherein the first separating apparatus is capable of (i.e., is . configured for) separating proteins based on a first physical property, and iii) a second separating apparatus, wherein the second separating apparatus is a liquid phase separating apparatus and wherein the second separating apparatus is capable of (i.e., is

25   configured for) separating proteins based on a second physical property; treating the sample with the first separating apparatus to produce a first separated protein sample; treating the first separated protein sample with the second separating apparatus to produce a second separated protein sample; and characterizing the second separated protein sample under conditions such that the first and the second physical properties

30   of at least a portion of the plurality of proteins are analyzed. In some embodiments,

the characterizing comprises quantitating the first physical property and the second physical property for two or more proteins in the second protein sample. In other preferred embodiments, the characterizing comprises the step of analyzing at least a portion of the second separated protein sample by mass spectrometry. In yet another

5   embodiment, the characterizing comprises the step of determining the identity of at least one protein from the second separated protein sample with mass spectrometry.

The present invention also provides a method for comparing protein expression patterns comprising providing: i) first and second samples comprising a plurality of proteins, ii) a first separating apparatus, wherein the first separating apparatus is

10   capable of (i.e., is configured for) separating proteins based on a first physical property; and iii) a second separating apparatus, wherein the second separating apparatus is a liquid phase separating apparatus and wherein the second separating apparatus is capable of separating proteins based on a second physical property; treating the first and second samples with the first separating apparatus to produce first

15   and second separated protein samples; treating the first and second separated protein samples with the second separating apparatus to produce third and fourth separated protein samples; and comparing the first and said second physical properties of the third separated protein sample with the first and second physical properties of the fourth separated protein sample. In some embodiments, the first and second samples

20   are combined into a single sample prior to step b) (i.e., the samples are run together rather than in parallel or in sequence). In some embodiments, at least a portion of the proteins in the first sample comprise a first label and at least a portion of the proteins in the second sample comprises a second label. In some embodiments, the comparing comprises the step of analyzing at least a portion of the third and the fourth separated

25   protein samples by mass spectrometry.

The present invention also provides a system comprising: a first separating apparatus, wherein the first separating apparatus is capable of (i.e., is configured for) separating proteins based on a first physical property; a first delivery apparatus capable of (i.e., configured for) receiving separated protein from the first separating apparatus;

30   a second separating apparatus wherein the second separating apparatus is a liquid phase

separating apparatus, wherein the second separating apparatus is capable of (*i.e.*, is configured for) separating proteins based on a second physical property, and wherein the second separating apparatus is capable of (*i.e.*, configured for) receiving proteins from the first delivery apparatus; a detection system capable of (*i.e.*, configured for)

5   detecting proteins produced by the second separating apparatus; a processor connected to the detection system, wherein the processor produces a data representation of the proteins produced by the second separating apparatus; and a display system capable of (*i.e.*, configured for) displaying the data representation under conditions such that the first and second physical properties of at least a portion of the plurality of proteins are

10   revealed. In some embodiments, the system further comprises a second delivery apparatus capable of (*i.e.*, configured for) receiving separated protein from the second separating apparatus; and a mass spectrometry apparatus capable of (*i.e.*, configured for) receiving protein from the second delivery apparatus.

The present invention also provides an method, comprising: providing: i) a

15   sample comprising a plurality of proteins, ii) a first separating apparatus capable of separating proteins based on a first physical property; iii) a second separating apparatus capable of separating proteins based on a second physical property; iv) a mass spectroscopy apparatus capable of separating proteins based on mass; and v) an automated sample handling device; and treating the sample with the first separating

20   apparatus to produce a first separated protein sample collected from the first separating apparatus in a plurality of fractions, each of the fractions defined by a distinct physical property; transferring the first separated protein sample to the second separating apparatus using the automated sample handling device; treating the first separated protein sample with the second separating apparatus to produce a second separated

25   protein sample; transferring the second separated protein sample to the mass spectroscopy apparatus using the automated sample handling device; and analyzing the second separated protein sample with the mass spectroscopy apparatus to produce a 3-dimensional protein map.

In some embodiments, the method further comprises a centralized control network operably linked to the automated sample handling device, the first separating apparatus, the second separating apparatus, and the mass spectroscopy apparatus. In some preferred embodiments, the centralized control network is configured to control the automated sample handling device, the first separating apparatus, the second separating apparatus, and the mass spectroscopy apparatus. In some embodiments, the centralized control network comprises computer memory and a computer processor.

In some embodiments, the method further comprises a solid phase extraction apparatus. In some embodiments, the first separated sample is treated with the solid phase extraction apparatus to treating with the second apparatus. In some embodiments, the automated sample handling device comprises a switchable, multi-channel valve.

In some embodiments, the sample comprising a plurality of proteins further comprises a buffer, wherein the plurality of proteins are solubilized in the buffer and wherein the buffer is compatible with the first separating apparatus, the second separating apparatus, and the mass spectroscopy apparatus. In some preferred embodiments, the buffer comprises a compound of the formula n-octyl SUGARpyranoside. The present invention is not limited to any particular buffer of the formula n-octyl SUGARpyranoside. Indeed, a variety of buffers are contemplated, including but not limited to, n-octyl ß-D-glucopyranoside and n-octyl ß-D-galactopyranoside. In some embodiments, the sample comprises a cell lysate.

In some embodiments, the first separating apparatus comprises a liquid phase separating apparatus. In some embodiments, the liquid phase separating apparatus in a ion exchange separating apparatus. In some embodiments, the second separating apparatus comprises a reverse phase HPLC separating apparatus. In some embodiments, the reverse phase HPLC comprises non-porous reverse phase HPLC.

In some embodiments, prior to said analyzing the second separated protein sample by mass spectroscopy; the second treated sample is separated into a first portion and a second portion; and the second portion is subjected to enzymatic digestion. In some embodiments, the mass spectrometry step comprises analyzing the

second separated protein sample by ESI oa TOF/MS. The present invention is not limited to any particular mass spectroscopy technique. Indeed, a variety of mass spectroscopy techniques are contemplated, including but not limited to, ion trap mass spectrometry, ion trap/time-of-flight mass spectrometry, quadrupole and triple

5   quadrupole mass spectrometry, Fourier Transform (ICR) mass spectrometry, and magnetic sector mass spectrometry.

In some embodiments, the 3-dimensional protein map comprises a first dimension representing protein charge, a second dimension representing protein hydrophobicity, and a third dimension representing protein mass. In some

10   embodiments, a second protein sample comprising a second plurality of proteins is analyzed. In some embodiments, the 3-D protein map comprises a comparison of protein expression patterns between the first protein sample and the second protein sample.

The present invention also provides a centralized control network operably

15   linked to a system, the system comprising: a first separating apparatus; an automated sample handling device; a second separating apparatus operably linked to the first separating apparatus and the sample handling device, wherein the second separating apparatus is configured to receive proteins from the first separating apparatus; a mass spectrometry apparatus operably linked to the second separating apparatus and the

20   sample handling device; wherein the mass spectroscopy apparatus is configured to receive proteins from the second separating apparatus; and wherein the centralized control network is capable of controlling the sample handling device, the first separating apparatus, the second separating apparatus; and the mass spectrometry apparatus.

25   In some embodiments, the centralized control network comprises computer memory and a computer processor. In some preferred embodiments, the centralized control network is capable of producing a 3-dimensional data representation of separated proteins analyzed by the mass spectrometry apparatus. In some embodiments, the automated sample handling device comprises a switchable, multi-channel valve.

In some embodiments, the system further comprises a display system capable of displaying the 3-D data representation. In some embodiments, the 3-dimensional data representation comprises a first dimension representing protein charge, a second dimension representing protein hydrophobicity, and a third dimension representing
5   protein mass.

In some embodiments, the first separating apparatus comprises a liquid phase separating apparatus. In some embodiments, the liquid phase separating apparatus is a ion exchange separating apparatus. In some embodiments, the second separating apparatus comprises a reverse phase HPLC separating apparatus. In some
10   embodiments, the reverse phase HPLC separating apparatus comprises a non-porous reverse phase HPLC apparatus.

DESCRIPTION OF THE FIGURES

Figure 1 shows an example 2-D protein display using Isoelectric Focusing Non-Porous Reverse Phase HPLC (IEF-NP RP HPLC) separation of human
15   erythroleukemia cell lysate proteins in one embodiment of the present invention.

Figure 2 shows a zoom area of a portion of the display in Figure 1 (pI = 4.2 to 7.2 and tR = 6.0 to 9.0) (right panel showing banding patterns) and a corresponding example of linked HPLC data (left panel showing peaks).

Figure 3 shows a quantification of rotofor fractions in one embodiment of the
20   present invention.

Figure 4 shows NP RP HPLC separation from a Rotofor fraction of HEL cell lysate in one embodiment of the present invention.

Figure 5A and 5B show short (5A) and long (5B) NP RP HPLC separation gradient times for a rotofor fraction of HEL cell lysate in one embodiment of the
25   present invention.

Figure 6 shows an example of Coomassie blue stained 2-D PAGE separation of HEL cell lysate proteins.

Figure 7 shows a direct side-by-side comparison of IEF-NP RP HPLC (four lanes on the left) with 1-D SDS PAGE (four lane on the right) for several Rotofor fractions in certain embodiments of the present invention.

Figures 8A and 8B show MALDI-TOF MS tryptic peptide mass maps for α-enolase isolated by IEF-NP RP HPLC (8A) and by 2-D PAGE (8B).

Figure 9 shows a 2D protein image of Isoelectric Focusing - Non-porous RP HPLC - ESI oa TOF/MS (IEF-NPS RP HPLC-ESI oa TOF/MS) separation of human erythroleukemia cell lysate proteins.

Figure 10 shows a zoom of the 2D protein image from Figure 9 of 35 kDa to 52 kDa mass range.

Figure 11A and 11B show actin multiply charged umbrella with MaxEnt deconvoluted molecular weight mass spectrum. The umbrella for beta and gamma actin is shown in Figure11A, each form of actin being labeled with the charge state. Figure 11B shows the resulting molecular weight mass spectrum for actin where the two forms of actin are separated.

Figure 12 shows combined protein molecular weight mass spectrum from a Rotofor fraction shown in traditional peak format.

Figure 13 shows a zoom of 2D protein image from Figure 9 of 5 kDa to 40 kDa mass range.

Figure 14 shows a chromatofocusing profile of MCF-10A whole cell lysate.

Figures 15A, B, and C show NP-RP-HPCL-ESI-oaTOF TIC (total ion count) profile of three sample fractions identified in Figure 14.

Figure 16 shows an integrated and deconvoluted TIC profile of the three sample fractions from Figure 15, as generated with MaxEnt1 software.

Figure 17 shows the anion exchange profile of Siberian Permafrost whole cell lysate of sample 23-9-25.

Figures 18A and 18B show the NP-RP-HPLC-ESI-oaTOF TIC profile of two fractions from Figure 17.

GENERAL DESCRIPTION OF THE INVENTION

The present invention relates to multi-phase protein separation methods capable of resolving large numbers of cellular proteins, including methods for efficiently facilitating the transfer of protein samples between separation phases. The methods of the present invention provide protein profile maps for imaging and comparing protein expression patterns. The present invention provides alternatives to traditional 2-D gel separation methods for the screening of protein profiles. Many limitations of traditional 2-D PAGE arise from its use of the gel as the separation media. The present invention provides alternative media for the separation that offer significant advantages over 2-D PAGE techniques. For example, in some embodiments, the present invention provides methods that use two dimensional separations, where the second dimensional separation occurs in the liquid phase, rather than 2-D PAGE techniques where the final separation occurs in gel.

The present invention provides systems and methods for protein separation and mapping that are highly efficient, amenable to automation, and provide detailed resolution. For example, in some methods of the present invention, proteins are separated according to their pI, using isoelectric focusing (IEF) (*e.g.*, in the Rotofor); according to their hydrophobicity using non-porous reverse phase HPLC (NPS RP HPLC); and according to mass using ESI oa TOF/MS or other mass spectrometry techniques. The present invention further provides novel techniques for eluting proteins from a separation apparatus (*e.g.*, the first phase separation apparatus). For example, in one embodiment of the present invention, the proteins eluted from the first dimension are "peeled off" from the column according to their pH, either one pH unit or fraction thereof, at a time. In some embodiments, these focused liquid fractions are then separated according to their hydrophobicity and size (or other desired properties) in the second dimension. Liquid fractions from, for example, NP-RP-HPLC can be conveniently analyzed directly on-line using mass spectrometry (*e.g.*, ESI-oaTOF) to obtain their molecular weight and relative abundance, which provides a third dimension. As a result, a virtual 2-D protein image is created and is analogous to a 2-D gel image.

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Experiments conducted during the development of the present invention have demonstrated that these methods are capable of separating large numbers of proteins. The 2-D image of these proteins, analogous to that of a 2-D gel, can be generated for the purpose of observing distinctive patterns from a particular cell line. This protein

5   pattern provides relative quantitative information, high mass resolution and high accuracy pI and mass values. Given that the intensity, mass and pI values are reproducible, one can study differential expression of proteins where the resulting 2-D images from different cells, tissues, or samples can be quantitatively compared to identify points of interest. Furthermore, automation and speed of analysis are greatly

10   facilitated given that the proteins remain in the liquid phase throughout the separation. The method, abbreviated IEF-NPS RP HPLC-ESI oa TOF/MS is shown to be a viable alternative for the separation of complex protein mixtures and the generation of high-resolution 2-D images of cellular protein expression.

In some embodiments of the present invention, proteins are separated in a first

15   dimension using any of a large number of protein separation techniques including, but not limited to, ion exclusion, ion exchange, normal/reversed phase partition, size exclusion, ligand exchange, liquid/gel phase isoelectric focusing, and adsorption chromatography. In some preferred embodiments of the present invention, the first dimension is a liquid phase separation method. The sample from the first separation is

20   passed through a second dimension separation. In preferred embodiments of the present invention, the second dimension separation is conducted in liquid phase. The products from the second dimension separation are then characterized. For example, in preferred embodiments, the products of the second separation step are detected and displayed in a 2-D format based on the physical properties of the proteins that were

25   distinguished in the first and second separation steps (e.g., under conditions such that the first and the second physical properties are revealed for at least a portion of the proteins). The products may be further analyzed, for example, by mass spectrometry to determine the mass and/or identity of the products or a subset of the products. In these embodiments, a three dimensional characterization can be applied (i.e., based on

30   the physical properties of the first two separation steps and the mass spectrometry

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data). It is contemplated that other protein processing steps can be conducted at any stage of the process.

In certain embodiments of the present invention, the steps are combined in an automated system. In preferred embodiments, each of the steps is automated. For

5   example, the present invention provides a system that includes each of the separation and detection elements in operable combination so that a protein sample is applied to the system and the user receives expression map displays or other desired data output. To achieve automation, in preferred embodiments, the products of each step should be compatible with the subsequent step or steps.

10   In one illustrative embodiment of the present invention proteins are separated according to their pI, using isoelectric focusing (IEF) in a Rotofor and according to their hydrophobicity and molecular weight using NP RP HPLC. This combined separation method is abbreviated IEF-NP RP HPLC. When coupled with mass spectrometry (MS) this technique becomes three-dimensional and allows for the

15   creation of a protein map that tells the pI and the molecular weight of the proteins in question. This information can be plotted in an image that also depicts protein abundance. The end result is a high-resolution image showing a complex pattern of proteins separated by pI and molecular weight and indicating relative protein abundances. This image can be used to determine how the proteins in a given cell line

20   or tissue may change due to some disease state, pharmaceutical treatment, natural or induced differentiation, or change in environmental conditions. The image allows the observer to determine changes in pI, molecular weight, and abundance of any protein in the image. When interfaced to MS the identity of any target protein may also be obtained via enzymatic digests and peptide mass map analyses. In addition, this

25   technique has the advantage of very high loadability (e.g., 1 gram) such that the lower abundance proteins may be detected.

In traditional 2-D PAGE separation and display techniques, the second phase separation is conducted in a gel (i.e., not a liquid phase) and the proteins are separated and detected by differences in molecular weight. In contrast, in some embodiments of

30   the present invention, the second phase separation is conducted in liquid phase. The

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products of the second phase separation techniques of the present invention are much more amenable to further characterization and to interpretation of data produced from the second phase. For example, in some embodiments of the present invention, the second phase is conducted using HPLC where the separated protein products are

5   readily detected as peak fractions and interpreted and displayed in two dimensions by a computer based on the physical properties of the first and second separation steps. The products of HPLC separation, being in the liquid phase, are readily used in further detection steps (e.g., mass spectrometry). The methods of the present invention, as compared to traditional 2-D PAGE, allow more sample to be analyzed, are more

10   efficient, facilitate automation, and allow for the analysis of proteins that are not detectable with 2-D PAGE.

For example, in one illustrative embodiment of the present invention, the protein profile of human erythroleukemia (HEL) cells has been analyzed using the methods of the present invention as well as traditional gel based methods for

15   comparison purposes. Two-dimensional images were generated representing each of the separation methods used. Proteins were separated and then collected using both the IEF-NP RP HPLC of the present invention and 2-D PAGE methods. These proteins were then enzymatically digested and the peptide mass maps were determined by MALDI-TOF MS (if a protein cannot be unambiguously identified by this method,

20   further analysis is made by any number of techniques including, but not limited to, LC/MS-MS, PSD-MALDI, NMR, Western blotting, Edman sequence analysis and mass spectrometry can help with further analysis of proteins [See e.g., Yates, J. Mass Spec., 33:1 (1998); Chen et al., Rap. Comm. Mass Spec., 13:1907 (1999); Neubauer and Mann, Anal. Chem. 71:235 (1999); Zugaro et al., Electrophoresis 19:867 (1998);

25   Immler et al., Electrophoresis 19:1015 (1998); Reid et al., Electrophoresis 19:946 (1998); Rosenfeld, et al., Anal. Biochem., 203:173 (1992); Matsui et al., Electrophoresis 18:409 (1997); Patterson and Aebersold, Electrophoresis 16:1791 (1995)]).

In some embodiments, the proteins were tentatively identified using MS-Fit to search the peptide mass maps against the Swiss and NCBInr protein databases. This work demonstrated that a large number of proteins, with a useful mass range, were separated using the methods of the present invention and that a 2-D image of these

5   proteins was reproducibly generated for the purpose of observing distinctive patterns that are associated with a particular cell line. The methods of the present invention allowed for the detection of proteins not observed with the 2-D PAGE technique. Automation and speed of analysis are also greatly facilitated given that the proteins remain in the liquid phase throughout the separation.

10   In some embodiments, the present invention provides an automated protein separation and characterization system. The system is fully integrated and transfers and coordinates multi-phase, orthogonal separation methods. In some embodiments, the information is transferred by the automated system to software for the generation of multi-dimensional protein maps. Automation provides increased speed, efficiency,

15   and sample recovery while eliminating potential sources of contamination and sample loss.

Thus, the methods of the present invention are shown to be an advantageous technique for the generation of images of protein expression profiles as well as for the collection of individual proteins for further analyses. These capabilities allow one to

20   monitor changes in protein expression that are linked to differentiation pathways as well as particular conditions such as cancer (See e.g., Hanash, Advances in Electrophoresis; Chrambach, A., Editor, pp 1-44 [1998]), cell aging (See e.g., Steller, Science 267:1445 [1995]), the response of cells to environmental insult (See e.g., Welsh et al., Biol. Reprod., 55:141 [1996]), or the response of cells to some

25   pharmaceutical agent. Having identified significant changes in protein expression, one can then further analyze proteins of interest to determine their identity and whether they have been altered from their expected structure by sequence changes or post-translational modifications.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the term "multiphase protein separation" refers to protein
separation comprising at least two separation steps. In some embodiments, multiphase protein separation refers to two or more separation steps that separate proteins based on different physical properties of the protein (*e.g.*, a first step that separates based on protein charge and a second step that separates based on protein hydrophobicity).

As used herein, the term "protein profile maps" refers to representations of the
protein content of a sample. For example, "protein profile map" includes 2-dimensional displays of total protein expressed in a given cell. In some embodiments, protein profile maps may also display subsets of total protein in a cell. Protein profile maps may be used for comparing "protein expression patterns" (*e.g.*, the amount and identity of proteins expressed in a sample) between two or more samples. Such
comparing find use, for example, in identifying proteins that are present in one sample (*e.g.*, a cancer cell) and not in another (*e.g.*, normal tissue), or are over- or under-expressed in one sample compared to the other.

As used herein, the term "separating apparatus capable of separating proteins based on a physical property" refers to compositions or systems capable of separating
proteins (*e.g.*, at least one protein) from one another based on differences in a physical property between proteins present in a sample containing two or more protein species. For example, a variety of protein separation columns and composition are contemplated including, but not limited to ion exclusion, ion exchange, normal/reversed phase partition, size exclusion, ligand exchange, liquid/gel phase
isoelectric focusing, and adsorption chromatography. These and other apparatuses are capable of separating proteins from one another based on their size, charge, hydrophobicity, and ligand binding affinity, among other properties. A "liquid phase" separating apparatus is a separating apparatus that utilizes protein samples contained in liquid solution, wherein proteins remain solubilized in liquid phase during separation

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and wherein the product (*e.g.*, fractions) collected from the apparatus are in the liquid phase. This is in contrast to gel electrophoresis apparatuses, wherein the proteins enter into a gel phase during separation. Liquid phase proteins are much more amenable to recovery/extraction of proteins as compared to gel phase. In some embodiments,

5   liquid phase proteins samples may be used in multi-step (*e.g.*, multiple separation and characterization steps) processes without the need to alter the sample prior to treatment in each subsequent step (*e.g.*, without the need for recovery/extraction and resolubilization of proteins).

As used herein, the term "displaying proteins" refers to a variety of techniques

10   used to interpret the presence of proteins within a protein sample. Displaying includes, but is not limited to, visualizing proteins on a computer display representation, diagram, autoradiographic film, list, table, chart, etc. "Displaying proteins under conditions that first and second physical properties are revealed" refers to displaying proteins (*e.g.*, proteins, or a subset of proteins obtained from a separating apparatus)

15   such that at least two different physical properties of each displayed protein are revealed or detectable. For example, such displays include, but are not limited to, tables including columns describing (*e.g.*, quantitating) the first and second physical property of each protein and two-dimensional displays where each protein is represented by an X,Y locations where the X and Y coordinates are defined by the

20   first and second physical properties, respectively, or vice versa. Such displays also include multi-dimensional displays (*e.g.*, three dimensional displays) that include additional physical properties.

As used herein, "characterizing protein samples under conditions such that first and second physical properties are analyzed" refers to the characterization of two or

25   more proteins, wherein two different physical properties are assigned to each analyzed (*e.g.*, displayed, computed, etc.) protein and wherein a result of the characterization is the categorization (*i.e.*, grouping and/or distinguishing) of the proteins based on these two different physical properties. For example, in some embodiments, two proteins are separated based on isoelectric point and hydrophobicity.

As used herein, the term "comparing first and second physical properties of separated protein samples" refers to the comparison of two or more protein samples (or individual proteins) based on two different physical properties of the proteins within each protein sample. Such comparing includes grouping of proteins in the samples

5   based on the two physical properties and comparing certain groups based on just one of the two physical properties (*i.e.*, the grouping incorporates a comparison of the other physical property).

As used herein, the term "delivery apparatus capable of receiving a separated protein from a separating apparatus" refers to any apparatus (*e.g.*, microtube, trough,

10   chamber, etc.) that receives one or more fractions or protein samples from a protein separating apparatus and delivers them to another apparatus (*e.g.*, another protein separation apparatus, a reaction chamber, a mass spectrometry apparatus, etc.).

As used herein, the term "detection system capable of detecting proteins" refers to any detection apparatus, assay, or system that detects proteins derived from a

15   protein separating apparatus (*e.g.*, proteins in one or fractions collected from a separating apparatus). Such detection systems may detect properties of the protein itself (*e.g.*, UV spectroscopy) or may detect labels (*e.g.*, fluorescent labels) or other detectable signals associated with the protein. The detection system converts the detected criteria (*e.g.*, absorbance, fluorescence, luminescence etc.) of the protein into

20   a signal that can be processed or stored electronically or through similar means (*e.g.*, detected through the use of a photomultiplier tube or similar system).

As used herein, the term "buffer compatible with an apparatus" and "buffer compatible with mass spectrometry" refer to buffers that are suitable for use in such apparatuses (*e.g.*, protein separation apparatuses) and techniques. A buffer is suitable

25   where the reaction that occurs in the presence of the buffer produces a result consistent with the intended purpose of the apparatus or method. For example, a buffer compatible with a protein separation apparatus solubilizes the protein and allows proteins to be separated and collected from the apparatus. A buffer compatible with mass spectrometry is a buffer that solubilizes the protein or protein fragment and

allows for the detection of ions following mass spectrometry. A suitable buffer does not substantially interfere with the apparatus or method so as to prevent its intended purpose and result (*i.e.*, some interference may be allowed).

As used herein, the term "automated sample handling device" refers to any device capable of transporting a sample (*e.g.*, a separated or un-separated protein sample) between components (*e.g.*, separating apparatus) of an automated method or system (*e.g.*, an automated protein characterization system). An automated sample handling device may comprise physical means for transporting sample (*e.g.*, multiple lines of tubing connected to a multi-channel valve). In some embodiments, an automated sample handling device is connected to a centralized control network.

As used herein, the term "switchable multi channel valve" refers to a valve that directs the flow of liquid through an automated sample handling device. The valve preferably has a plurality of channels (*e.g.*, 2 or more, and preferably 4 or more, and more preferably, 6 or more). In addition, in some embodiments, flow to individual channels is "switched" on an off. In some embodiments, valve switching is controlled by a centralized control system. A switchable multi-channel valve allows multiple apparatus to be connected to one automated sample handler. For example, sample can first be directed through one apparatus of a system (*e.g.*, a first chromatography apparatus). The sample can then be directed through a different channel of the valve to a second apparatus (*e.g.*, a second chromatography apparatus).

As used herein, the terms "centralized control system" or "centralized control network" refer to information and equipment management systems (*e.g.*, a computer processor and computer memory) operable linked to multiple devices or apparatus (*e.g.*, automated sample handling devices and separating apparatus). In preferred embodiments, the centralized control network is configured to control the operations or the apparatus an device linked to the network. For example, in some embodiments, the centralized control network controls the operation of multiple chromatography apparatus, the transfer of sample between the apparatus, and the analysis and presentation of data.

As used herein, the terms "computer memory" and "computer memory device" refer to any storage media readable by a computer processor. Examples of computer memory include, but are not limited to, RAM, ROM, computer chips, digital video disc (DVDs), compact discs (CDs), hard disk drives (HDD), and magnetic tape.

5   As used herein, the term "computer readable medium" refers to any device or system for storing and providing information (*e.g.*, data and instructions) to a computer processor. Examples of computer readable media include, but are not limited to, DVDs, CDs, hard disk drives, magnetic tape and servers for streaming media over networks.

10   As used herein, the terms "processor" and "central processing unit" or "CPU" are used interchangeably and refers to a device that is able to read a program from a computer memory (*e.g.*, ROM or other computer memory) and perform a set of steps according to the program.

       As used herein, the term "directly feeding" a protein sample from one apparatus

15   to another apparatus refers to the passage of proteins from the first apparatus to the second apparatus without any intervening processing steps. For example, a protein that is directly fed from a protein separating apparatus to a mass spectrometry apparatus does not undergo any intervening digestion steps (*i.e.*, the protein received by the mass spectrometry apparatus is undigested protein).

20   As used herein, the term "sample" is used in its broadest sense. In one sense it can refer to a cell lysate. In another sense, it is meant to include a specimen or culture obtained from any source, including biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products (*e.g.*, plasma and

25   serum), saliva, urine, and the like and includes substances from plants and microorganisms. Environmental samples include environmental material such as surface matter, soil, water, and industrial samples. These examples are not to be construed as limiting the sample types applicable to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel multi-dimensional separation method that is capable of resolving large numbers of cellular proteins. The following discussion is provided in four sections: I) two-phase separation techniques; II) improved elution techniques; III) mass spectroscopic analysis and 2-D display systems and methods; and IV) automated 3D HPLC/MC methods for rapid protein characterization.

I)   Two-Phase Separation Techniques

The first dimension separates proteins based on a first physical property. For example, in some embodiments of the present invention proteins are separated by pI using isoelectric focusing in the first dimension (See e.g., Righetti, Laboratory Techniques in Biochemistry and Molecular Biology; Work, T. S.; Burdon, R. H., Elsevier: Amsterdam, p 10 [1983]). However, the first dimension may employ any number of separation techniques including, but not limited to, ion exclusion, ion exchange, normal/reversed phase partition, size exclusion, ligand exchange, liquid/gel phase isoelectric focusing, and adsorption chromatography. In some embodiments (e.g., some automated embodiments), it is preferred that the first dimension be conducted in the liquid phase to enable products of the separation step to be fed directly into a second liquid phase separation step.

The second dimension separates proteins based on a second physical property (i.e., a different property than the first physical property) and is preferably conducted in the liquid phase (e.g., liquid-phase size exclusion). For example, in some embodiments of the present invention proteins are separated by hydrophobicity using non-porous reversed phase HPLC in the second dimension (See e.g., Liang et al., Rap. Comm. Mass Spec., 10:1219 [1996]; Griffin et al., Rap. Comm. Mass Spec., 9:1546 [1995]; Opiteck et al., Anal. Biochem. 258:344 [1998]; Nilsson et al., Rap. Comm. Mass Spec., 11:610 [1997]; Chen et al., Rap. Comm. Mass Spec., 12:1994 [1998]; Wall et al., Anal. Chem., 71:3894 [1999]; Chong et al., Rap. Comm. Mass Spec.,

13:1808 [1999]). This method provides for exceptionally fast and reproducible high-resolution separations of proteins according to their hydrophobicity and molecular weight. The non-porous (NP) silica packing material used in these reverse phase (RP) separations eliminates problems associated with porosity and low recovery of larger

5   proteins, as well as reducing analysis times by as much as one third. Separation efficiency remains high due to the small diameter of the spherical particles, as does the loadability of the NP RP HPLC columns. However, the second dimension may employ any number of separation techniques. For example, in one embodiment, 1-D SDS PAGE lane gel is used. Having the second dimension conducted in the liquid

10   phase facilitates efficient analysis of the separated proteins and enables products to be fed directly into additional analysis steps (*e.g.*, directly into mass spectrometry analysis).

In certain embodiments of the present invention, proteins obtained from the second separation step are mapped using software (available from Dr. Stephen J.

15   Parus, University of Michigan, Department of Chemistry, 930 N. University Ave., Ann Arbor, MI 48109-1055) in order to create a protein pattern analogous to that of the 2-D PAGE image--although based on the two physical properties used in the two separation steps rather than by a second gel-based size separation technique. In some embodiments, RP HPLC peaks are represented by bands of different intensity in the 2-

20   D image, according to the intensity of the peaks eluting from the HPLC. In some embodiments, peaks are collected as the eluent of the HPLC separation in the liquid phase.

In some embodiments, the proteins collected from the second dimension were identified using proteolytic enzymes, MALDI-TOF MS and MSFit database searching.

25   In an example using human erythroleukemia cell lysate, using IEF-NP RP HPLC, approximately 700 bands were resolved in a pI range from 3.2 to 9.5 and 38 different proteins with molecular weights ranging from 12 kDa to 75 kDa were identified. In comparison to a 2-D gel separation of the same human erythroleukemia (HEL) cell line lysate, the IEF-NP RP HPLC produced improved resolution of low mass and basic

30   proteins. In addition, the proteins remained in the liquid phase throughout the

separation, thus making the entire procedure highly amenable to automation and high throughput.

Certain preferred embodiments are described in detail below. These illustrative examples are not intended to limit the scope of the invention. For example, although

5   the examples are described using human tissues and samples, the methods and apparatuses of the present invention can be used with any desired protein samples including samples from plants and microorganisms.

A.   IEF-NP RP HPLC Method

The following description provides certain preferred embodiments for

10   conducting isoelectric separation (first dimension) and NP RP HPLC separation (second dimension) according to the methods of the present invention.

1.   IEF Separation

Proteins are extracted from cells using a lysis buffer. To facilitate an efficient process, this lysis buffer should be compatible with the downstream separation and

15   analysis steps (*e.g.*, NP RP HPLC and MALDI-TOF-MS) to allow direct use of the products from each step into subsequent steps. Such a buffer is an important aspect of automating the process. Thus, the preferred buffer should meet two criteria: 1) it solubilizes proteins and 2) it is compatible with each of the steps in the separation/analysis methods. Although the present invention provides suitable buffers

20   for use in the particular method configurations described below, one skilled in the art can determine the suitability of a buffer for any particular configuration by solubilizing protein sample in the buffer. If the buffer solubilizes the protein, the sample is run through the particular configuration of separation and detection methods desired. A positive result is achieved if the final step of the desired configuration produces

25   detectable information (*e.g.*, ions are detected in a mass spectrometry analysis). Alternately, the product of each step in the method can be analyzed to determine the presence of the desired product (*e.g.*, determining whether protein elutes from the separation steps).

After extraction in the lysis buffer, proteins are initially separated in a first dimension. The goal in this step is that the proteins are isolated in a liquid fraction that is compatible with subsequent NP RP HPLC and mass spectrometry steps. In these embodiments, n-octyl ß-D-glucopyranoside (OGl, from Sigma) is used in the

5   buffer. *n-octyl ß-D-glucopyranoside is one of the few detergents that is compatible with both NP RP HPLC and subsequent mass spectrometry analyses.* It is contemplated that detergents of the formula n-octyl SUGARpyranoside find use in these embodiments. The lysis buffer utilized was 6M urea, 2M thiourea, 1.0 % n-octyl ß-D-glucopyranoside, 10 mM dithioerythritol and 2.5 % (w/v) carrier ampholytes (3.5

10   to 10 pI)). After extraction, the supernatant protein solution is loaded to a device that can separate the proteins according to their pI by isoelectric focusing (IEF). Here the proteins are solubilized in a running buffer that again should be compatible with NP RP HPLC. A suitable running buffer is 6M urea, 2M thiourea, 0.5 % n-octyl ß-D-glucopyranoside, 10 mM dithioerythritol and 2.5 % (w/v) carrier ampholytes (3.5 to 10

15   pI).

Three exemplary devices that may be used for this step are:

a)   Rotofor

This device (Biorad) separates proteins in the liquid phase according to their pI (*See e.g.*, Ayala *et al.*, Appl. Biochem. Biotech. 69:11 [1998]). This device allows for

20   high protein loading and rapid separations that require only four to six hours to perform. Proteins are harvested into liquid fractions after a 5-hour IEF separation. These liquid fractions are ready for analysis by NP RP HPLC. This device can be loaded with up to 1 g of protein.

b)   Carrier Ampholyte based slab gel IEF separation with
25   **a whole gel eluter**

In this case the protein solution is loaded onto a slab gel and the proteins separate in to a series of gel-wide bands containing proteins of the same pI. These proteins are then harvested using a whole gel eluter (WGE, from Biorad). Proteins are

then isolated in liquid fractions that are ready for analysis by NP RP HPLC. This type of gel can be loaded with up to 20 mg of protein.

c)   IPG slab gel IEF separation with a whole gel eluter

Here the proteins are loaded onto a immobiline pI gradient slab gel and
5   separated into a series of gel-wide bands containing proteins of the same pI. These proteins are electro-eluted using the WGE into liquid fractions that are ready for analysis by NP RP HPLC. The IPG gel can be loaded with at least 60 mg of protein.

2.   Protein Separation by NP RP HPLC

Having obtained liquid fractions containing large amounts of pI-focused
10   proteins, the second dimension separation is non-porous RP HPLC. The present invention provides the novel combination of employing non-porous RP packing materials (Eichrom) with another RP HPLC compatible detergent (*e.g.*, n-octyl ß-D-galactopyranoside) to facilitate the multi-phase separation of the present invention. This detergent is also compatible with mass spectrometry due to its low molecular
15   weight. The use of these types of RP HPLC columns for protein separations as a second dimension separation after IEF in order to obtain a 2-D protein separation is a novel feature of the present invention. These columns are well suited to this task as the non-porous packing they contain provides optimal protein recovery and rapid efficient separations. It should be noted that though several detergents have been
20   mentioned thus far for increasing protein solubility while being compatible with RP HPLC there are many other different low molecular weight non-ionic detergents that could be used for this purpose. Several important features that allow the RP HPLC to work as a second dimension are as follows: The mobile phase should contain a low level of a non-ionic low molecular weight detergent such as n-octyl ß-D-
25   glucopyranoside or n-octyl ß-D-galactopyranoside as these detergents are compatible with RP HPLC and also with later mass spectrometry analyses (unlike many other detergents); the column should be held at a high temperature (around 60 °C); and the

column should be packed with non-porous silica beads to eliminate problems of protein recovery associated with porous packings.

3. Protein Detection and Identification via Mass Spectrometry

In some embodiments of the present invention, the products of the second separation step are further characterized using mass spectrometry. For example, the proteins that elute from the NP RP HPLC separation are analyzed by mass spectrometry to determine their molecular weight and identity. For this purpose the proteins eluting from the separation can be analyzed simultaneously to determine molecular weight and identity. A fraction of the effluent is used to determine molecular weight by either MALDI-TOF-MS or ESI oa TOF (LCT, Micromass) (*See e.g.,* U.S. Pat. No. 6,002,127). The remainder of the eluent is used to determine the identity of the proteins via digestion of the proteins and analysis of the peptide mass map fingerprints by either MALDI-TOF-MS or ESI oa TOF. The molecular weight 2-D protein map is matched to the appropriate digest fingerprint by correlating the molecular weight total ion chromatograms (TIC's) with the UV-chromatograms and by calculation of the various delay times involved. The UV-chromatograms are automatically labeled with the digest fingerprint fraction number. The resulting molecular weight and digest mass fingerprint data can then be used to search for the protein identity via web-based programs like MSFit (UCSF).

4. Automation

All of the above described steps are automated, for example, into one discrete instrument. In one illustrative embodiment, the first dimension is carried out by a Rotofor, with the harvested liquid fractions being directly applied to the second dimension non-porous RP HPLC apparatus through the appropriate tubing. The products from the second dimension separation are then scanned and the data interpreted and displayed as a 2-D representation using the appropriate computer hardware and software. Alternately, the products from the second dimension fractions are sent through the appropriate microtubing to a mass spectrometry pre-reaction

chamber where the samples are treated with the appropriate enzymes to prepare them
for mass spectrometry analysis. The samples are then analyzed by mass spectrometry
and the resulting data is received and interpreted by a processor. The output data
represents any number of desired analyses including, but not limited to, identity of the

5   proteins, mass of the proteins, mass of peptides from protein digests, dimensional
displays of the proteins based on any of the detected physical criteria (*e.g.*, size,
charge, hydrophobicity, etc.), and the like. In preferred embodiments, the proteins
samples are solubilized in a buffer that is compatible with each of the separation and
analysis units of the apparatus. Using the automated systems of the present invention

10   provides a protein analysis system that is an order of magnitude less expensive than
analogous automation technology for use with 2-D gels (*See e.g.*, Figeys and
Aebersold, J. Biomech. Eng. 121:7 [1999]; Yates, J. Mass Spectrom., 33:1 [1998]; and
Pinto *et al.*, Electrophoresis 21:181 [2000]).

5.   Software and Data Presentation

15   The data generated by the above listed techniques may be presented as 2-D
images much like the traditional 2-D gel image. In some embodiments, the
chromatograms, TIC's or integrated and deconvoluted mass spectra are converted to
ASCII format and then plotted vertically, using a 256 step gray scale, such that peaks
are represented as darkened bands against a white background. The scale could also

20   be in a color format. The image generated by this method provides information
regarding the pI, hydrophobicity, molecular weight and relative abundance of the
proteins separated. Thus the image represents a protein pattern that can be used to
locate interesting changes in cellular protein profiles in terms of pI, hydrophobicity,
molecular weight and relative abundance. Naturally the image can be adjusted to show

25   a more detailed zoom of a particular region or the more abundant protein signals can
be allowed to saturate thereby showing a clearer image of the less abundant proteins.
This information can be used to assess the impact of disease state, pharmaceutical
treatment, and environmental conditions. As the image is automatically digitized it
may be readily stored and used to analyze the protein profile of the cells in question.

Protein bands on the image can be hyper-linked to other experimental results, obtained

via analysis of that band, such as peptide mass fingerprints and MSFit search results.

Thus all information obtained about a given 2-D image, including detailed mass

spectra, data analyses, and complementary experiments (*e.g.*, immuno-affinity and

5   peptide sequencing) can be accessed from the original image.

The data generated by the above-listed techniques may also be presented as a

simple read-out. For example, when two or more samples are compared (*See*, Section

J, below), the data presented may detail the difference or similarities between the

samples (*e.g.*, listing only the proteins that differ in identity or abundance between the

10   samples). In this regard, when the differences between samples (*e.g.*, a control sample

and an experimental sample) are indicative of a given condition (*e.g.*, cancer cell, toxin

exposure, etc.), the read-out may simply indicate the presence or identity of the

condition. In one embodiment, the read-out is a simple +/- indication of the presence

of particular proteins or expression patterns associated with a specific condition that is

15   to be analyzed.

6.   IEF-NP RP HPLC in Operation

The IEF-NP RP HPLC image shown in Figure 1 is a digital representation of a

2-dimensional separation of a whole cell protein lysate from a human erythroleukemia

(HEL) cell line. This image is designed to offer the same advantages of pattern

20   recognition and protein profiling that may be obtained using a 2-D gel. The horizontal

and vertical dimensions are in terms of isoelectric point and protein hydrophobicity,

respectively. The isoelectric focusing step, performed using the Rotofor, resulted in 20

protein fractions ranging in pH from 3.2 to 9.5. These fractions were then injected

onto a non-porous reversed phase column for separation by HPLC and detection by

25   UV absorbance (214 nm). The resulting chromatograms were converted to ASCII

format and then plotted vertically, using a 256 step gray scale, such that peaks are

represented as darkened bands against a white background. Protein profiles may be

viewed in greater detail by using the zoom feature as shown in Figure 2 and/or by

selecting a particular Rotofor fraction and observing the NP RP HPLC chromatogram as shown in the left panel of Figure 2. The zoom and chromatogram image features provide a means to observe details in band patterns that may not be observable in the original image (*See*, Figure 1). In addition, because of the limitations of the 256 step

5   gray scale representation the band intensities in areas 1, 2 and 3 of Figure 1 were rescaled by a factor of 3 to better show the low abundance proteins. This was preferred since the presence of several high abundance protein bands may cause low intensity bands in some regions to be undetected. In Figure 1, the total peak area for each individual chromatogram was scaled to reflect the relative amount of protein that

10   was found in the original Rotofor fraction (*See*, Figure 3). The band intensities in different chromatograms can therefore be compared directly thus providing a true image of relative protein abundance in the cell lysate. The width of the Rotofor fraction columns was adjusted to represent their estimated pH range. The molecular weight of proteins observed by IEF-NP RP HPLC ranged from 12 kDa to 75 kDa.

15   Typical NP RP HPLC separations, as shown in Figure 4, resulted in 35 peaks in 10.5 minutes. The total number of peaks that could be observed from all 20 fractions is estimated to be approximately 700.

The gradient time (t_G) used in the above experiments is very short and a significant increase in peak capacity is expected with longer gradients. This is shown

20   using Rotofor fraction 17 where two separations were performed with gradient times of 10.5 minutes (*See*, Figure 5A) and 21 minutes (*See*, Figure 5B). With t_G = 10.5 minutes, the average peak width was 0.14 minutes and the peak capacity was therefore 75. The actual number of peaks resolved was 35. With t_G = 21 minutes the average peak width was 0.23 minutes and the peak capacity was therefore 91. The actual

25   number of peaks resolved was 51. Using the longer separation time with t_G = 21 minutes the total number of peaks observed should increase from 700 to 1000. However, it should be noted that when using mass spectrometric detection, that sufficient resolution should be available to ultimately resolve the same number of peaks without using a longer gradient time.

The proteins in a representative sampling of these peaks were identified using the traditional approach of enzymatic digestion, MALDI-TOF MS peptide mass analysis and MSFit database searching. The magnification of the IEF-NP RP HPLC image enables the viewer to perceive more bands than is possible to observe from the whole image. In addition, as shown in Figure 2, the viewer may select a particular band format chromatogram and observe the traditional peak format of the chromatogram in a window to the left of the image. This allows the observer to use the peak format chromatogram to find partially resolved peaks that may not be observable in the band format chromatogram. Five standard protein bands are shown in the left-most column where the masses range from 14.2 kDa up to 67 kDa. As RP HPLC separates proteins by hydrophobicity, these standards are not molecular weight markers as in a traditional 1-D gel. Rather, they are used to indicate the range of protein molecular weights that may be observed. Ten different proteins are labeled on the image although many more proteins were identified as shown in Table 1, below. In some embodiments of the present invention, where it is desired that certain proteins or classes of proteins are to be detected, the starting protein sample may be selectively labeled. After the proteins are passed through the separation step, detection of the proteins can be limited to those that contain the selective label.

B.   Protein Separation by 2-D SDS PAGE

The image in Figure 1 represents the IEF-NP RP HPLC separation of the HEL cell protein lysate and the image in Figure 6 represents the Coomassie blue (CBB) stained 2-D SDS PAGE separation of the same HEL cell line lysate. The pI range for this gel is the same as that used for the Rotofor separation and the molecular weight range is from 8 kDa to 140 kDa. As with the IEF-NP RP HPLC separation a representative sampling of the isolated proteins was identified using enzymatic digestion, MALDI-TOF MS and MSFit methods (*See e.g.*, Rosenfeld *et al.*, Anal. Biochem. 203:173 [1992]). For the target protein mass range of this study (10 kDa – 70 kDa) approximately 188 protein spots are observed on the CBB stained gel, 355 from the CBB stained polyvinylidene difluoride (PVDF) blot, and 652 from the silver

stained gel as estimated using BioImage 2D Analyzer Version 6.1 software (Genomic

Solutions). The total spot capacity for the 2-D gel separation is estimated to be 2100.

The proteins identified from the gel are labeled on the image and also shown in Table

2, below. An image of another 2-D gel separation of HEL cell proteins can be

5   observed via the Swiss-2DPAGE database (*See e.g.*, http://www.expasy.ch; Sanchez *et*

al., Electrophoresis 16:1131 [1995]). In addition, it is possible to view the latest

protein list for the HEL cell in which 19 protein entries are shown (*See e.g.*,

http://www.expasy.ch/cgi-bin/get-ch2d-table.pl).

Table 1. Thirty Eight Proteins Identified From HEL Cell IEF-NP RP HPLC Separation

Rotofor Fraction #	pH	Retention Time (min.)	Enzyme *	MWt / pI: database calculated	Swiss, NCBInr Accession #	Protein Name
3	4.20	5.34	trypsin	32575.2 / 4.64	P06748	NPM
3	4.20	6.20	trypsin	11665.0 / 4.42	P05387	60S RIBOSOMAL PROTEIN P2
3	4.20	6.91	trypsin	16837.7 / 4.09	P02593	CALMODULIN
3	4.20	10.15	trypsin	41737.0 / 5.29	P02570	BETA-ACTIN & GAMMA ACTIN
3	4.20	10.25	trypsin	61055.0 / 5.70	P10809	HSP-60
4	4.70	5.38	trypsin	32575.2 / 4.64	P06748	NPM
4	4.70	6.24	trypsin	35994.6 / 6.61	Q13011	ENOYL-COA HYDRATASE
4	4.70	7.07	trypsin	57914.2 / 7.95	P14786	PYRUVATE KINASE, M2
4	4.70	10.28	trypsin	61055.0 / 5.70	P10809	HSP-60
5	5.40	4.93	trypsin	22988.1 / 5.10	P52566	RHO GDI 2
5	5.40	10.15	trypsin	70898.4 / 5.38	P11142	HEAT SHOCK COGNATE 71 KD PROTEIN
8	5.60	4.99	trypsin	22988.1 / 5.10	P52566	RHO GDP-DISSOCIATION INHIBITOR 2
8	5.60	7.94	trypsin	69224.5 / 5.49	P23588	EIF-4B
8	5.80	10.35	trypsin	49831.3 / 4.79	P05217	TUBULIN BETA-2 CHAIN
9	5.80	6.90	trypsin	56782.7 / 5.99	P30101	ERP60
9	5.80	8.05	trypsin	17148.8 / 5.83	P15531	METASTASIS INHIBITION FACTOR NM23
9	5.80	8.50	trypsin	26669.6 / 6.45	P00938	TRIOSEPHOSPHATE ISOMERASE (TIM)
9	5.80	10.15	trypsin	41737.0 / 5.29	P02570	BETA-ACTIN & GAMMA ACTIN
11	6.20	5.62	trypsin	36926.7 / 6.37	5542020	(L32610) ribonucleoprotein
11	6.20	7.65	trypsin	33777.2 / 6.26	4885153	(X59656) CRKL
11	6.20	7.91	trypsin	22327.3 / 7.83	P04792	HEAT SHOCK 27
11	6.20	8.80	trypsin	74674.0 / 8.51	Q92935	EXOSTOSIN-L
11	6.20	9.22	trypsin	37374.9 / 5.85	P19883	FOLLISTATIN 1 AND 2 PRECURSOR
11	6.20	10.40	trypsin	47033.1 / 5.30	5032183	cargo selection protein TIP47
12	6.40	5.08	trypsin	13802.0 / 6.43	P49773	HINT
12	6.40	5.90	trypsin	70021.3 / 5.56	P54652	HEAT SHOCK 70 KD PROTEIN 2
12	6.40	7.48	trypsin	47169.2 / 7.01	P06733	ALPHA ENOLASE
12	6.40	8.12	trypsin	26669.6 / 6.45	P00938	TRIOSEPHOSPHATE ISOMERASE (TIM)
13	6.60	4.88	trypsin	48058.0 / 5.34	P05783	KERATIN, TYPE I CYTOSKELETAL 18
13	6.60	8.28	trypsin	62639.6 / 6.40	P31948	TRANSFORMATION-SENSITIVE PROTEIN
13	6.60	8.65	trypsin	34902.4 / 7.42	4505059	carcinoma-associated antigen GA733-2
15	7.00	4.70	trypsin	37429.9 / 8.97	P22626	NUCLEAR RIBONUCLEOPROTEINS A2/B1
15	7.00	8.70	trypsin	22391.6 / 8.41	P37802	SM22-ALPHA HOMOLOG
15	7.00	7.25	trypsin	47169.2 / 7.01	P06733	ALPHA ENOLASE
16	7.20	5.68	trypsin, Glu-C (E)	18012.6 / 7.68	P05092	PPIASE
16	7.20	6.89	trypsin	35940.7 / 7.18	P01861	IG GAMMA-4 CHAIN C REGION
16	7.20	7.24	trypsin	36053.4 / 8.57	P04406	GLYCERALDEHYDE 3-PHOSPHATE
16	7.20	7.45	trypsin, Glu-C (E)	47169.2 / 7.01	P06733	ALPHA ENOLASE
16	7.20	8.64	trypsin, Glu-C (E)	22391.6 / 8.41	P37802	SM22-ALPHA HOMOLOG
19	9.00	4.88	trypsin	38846.0 / 9.26	P09651	NUCLEAR RIBONUCLEOPROTEIN A1
19	9.00	5.13	trypsin	37429.9 / 8.97	P22626	NUCLEAR RIBONUCLEOPROTEINS A2/B1
19	9.00	5.85	trypsin	46987.1 / 7.58	P13929	BETA ENOLASE
19	9.00	7.47	trypsin	36053.4 / 8.57	P04406	GLYCERALDEHYDE 3-PHOSPHATE
19	9.00	8.70	trypsin	38604.2 / 7.58	P07355	ANNEXIN II
19	9.00	9.07	trypsin	22391.6 / 8.41	P37802	SM22-ALPHA HOMOLOG
19	9.00	10.53	trypsin	57221.6 / 9.22	P26599	PTB, NUCLEAR RIBONUCLEOPROTEIN I
20	9.50	4.46	trypsin, Glu-C (E)	38846.0 / 9.26	P09651	NUCLEAR RIBONUCLEOPROTEIN A1
20	9.50	4.67	trypsin, Glu-C (E)	37429.9 / 8.97	P22626	NUCLEAR RIBONUCLEOPROTEINS A2/B1
20	9.50	6.72	trypsin, Glu-C (E)	39420.2 / 8.30	P04075	FRUCTOSE-BISPHOSPHATE ALDOLASE A
20	9.50	7.06	trypsin	36053.4 / 8.57	P04406	GLYCERALDEHYDE 3-PHOSPHATE
20	9.50	7.39	trypsin, Glu-C (E)	47169.2 / 7.01	P06733	ALPHA ENOLASE
20	9.50	8.52	trypsin, Glu-C (E)	22391.6 / 8.41	P37802	SM22-ALPHA HOMOLOG
20	9.50	10.16	trypsin	44728.1 / 8.30	P00558	PHOSPHOGLYCERATE KINASE 1
20	9.50	10.35	trypsin	57221.6 / 9.22	P26599	PTB, NUCLEAR RIBONUCLEOPROTEIN I

* Note that all proteins labelled only with trypsin were not digested with Glu-C (E)

Table 2. Nine Proteins Identified From HEL Cell CBB 2-D Gel

Gel Spot I.D. Number	Enzyme	MWt / pI: database calculated	SwissProt Accession #	Protein Name
g1	trypsin	18012.6 / 7.68	P05092	PPIASE
g2	trypsin	26669.6 / 6.45	P00938	TRIOSEPHOSPHATE ISOMERASE (TIM)
g3	trypsin	26669.6 / 6.45	P00938	TRIOSEPHOSPHATE ISOMERASE (TIM)
g8	trypsin	29032.8 / 4.75	P12324	TROPOMYOSIN, CYTOSKELETAL TYPE (TM30-NM)
g10	trypsin	32575.2 / 4.64	P06748	NPM
g11	trypsin	41737.0 / 5.29	P02570	BETA-ACTIN
g12	trypsin	61055.0 / 5.70	P10809	HSP-60
g13	trypsin	56782.7 / 5.99	P30101	ERP60
g14	trypsin	47169.2 / 7.01	P06733	ALPHA ENOLASE

C.   IEF-NP RP HPLC versus 2-D SDS PAGE: Protein Loading and Quantification

Each separation method relies upon orthogonal mechanisms of separation generating a large number of isolated proteins. Protein profiles may be compared in terms of their pattern as well as the relative amounts of isolated proteins. It is shown, however, that the loadability of the liquid phase methods of the present invention greatly surpasses that of the gel phase.

The limit of detection for the gel method when stained with the silver stain is approximately 1 to 10 ng. The Coomassie blue stain can detect 100 ng of protein and the amount of protein in the spot can be quantified over 2.5 orders of magnitude. For the NP RP HPLC of standard proteins used in certain embodiments of the methods of the present invention, the limit of detection for the UV detector was 10 ng. The protein in the peak can be quantified from 10 ng up to 20 µg providing 3.1 orders of magnitude. Quantification of an HPLC peak involves integrating the peak to find the area. For the gel, the spots must first be digitized and then this image must be analyzed to determine the integrated optical density of each spot of interest. The sensitivity of the UV detector in embodiments of the present invention utilizing HPLC is competitive with the silver stain and quantification is much simpler. The limits of detection for both the silver stained gel and the HPLC UV peak detection are mass dependent. For the gel, resolution and sensitivity are proportional to the molecular weight of the protein. For IEF-NP RP HPLC, the resolution and sensitivity are inversely proportional to the molecular weight of the protein. The gel appears to provide improved results for both acidic proteins and proteins above 50 kDa whereas IEF-NP RP HPLC performs better with proteins in the basic region and proteins that are below 50 kDa (See e.g., Figure 1 and Figure 6). These results show the complementary nature of these two techniques where the gel and IEF-NP RP HPLC each provide important information of protein content.

In one experiment using the methods of the present invention, 23.5 mg of protein was loaded into the Rotofor, and after a five-hour IEF separation period fractions ranging from 2 to 4 mL were collected into polypropylene microtubes. The

amount of protein in the individual fractions ranged from 0.25 mg to 1.05 mg.
Summing the amounts of protein in each fraction led to the determination that a total
of 10.2 mg of protein was recovered from the Rotofor. This amount can be increased
by increasing the amount of non-ionic detergent in the Rotofor buffer above the

5   current 0.1% level as well as by the addition of thiourea. In contrast, the amount of
protein loaded on the 2-D gel in Figure 6 is 200 μg. The amount of protein that
actually makes it through the gel and focuses to a spot has not been quantified, relative
to the amount of protein that is actually loaded on the gel, though it is known that
many hydrophobic proteins are lost during the separation (Herbert, Electrophoresis

10   20:660 [1999]). The amount of protein that may theoretically be loaded on a gel
ranges from 5 μg up to 250 μg whereas for IEF-NP RP HPLC the initial loading of
protein may be as high as 1 gram. The amount of protein actually used to produce the
separation shown in Figure 1 is only a fraction of the amount initially loaded into the
Rotofor. The image in Figure 1 actually represents the separation of a total of 1 to 2

15   mg of protein though 10.2 mg of protein was recovered from the Rotofor. The
loading of the HPLC column being used currently could be increased though the peak
capacity may suffer. Alternatively a larger column could be used in series with the
smaller column to allow for higher loadability with no loss of separation efficiency
(See e.g., Wall et al., Anal. Chem., 71:3894 [1999]).

20   A 2-D gel provides a two dimensional separation from one initial loading of
the cell lysate. The intensities of different spots on the same gel are representative of
the relative protein abundances in the original lysate. However, in the IEF-NP RP
HPLC methods of the present invention the proteins are loaded for the IEF and the
HPLC separations so that the band intensities in the 2-D IEF-NP RP HPLC image

25   depend on the amount of protein loaded to the HPLC from each Rotofor fraction.
Since the amount of material in each Rotofor fraction is different, the total area of
each chromatogram was scaled to represent the total amount of protein that was
recovered for each Rotofor fraction (See, Figure 3). The result is that the protein band
intensities can be compared both within the Rotofor fraction and between the different

30   fractions.

In some embodiments of the present invention, 2-D gel techniques are used side-by-side with IEF-NP RP HPLC. In embodiments where specific proteins are desired for further characterization, the gel can provide information indicating which fraction obtained with IEF-NP RP HPLC contains the desired protein or proteins.

5 **D.   Isoelectric Focusing: Liquid vs. Gel Phase**

The principal concern with liquid phase IEF is that the protein is not isoelectrically focused as effectively as it would be in a gel due to diffusion of the protein in solution. In the case of α-enolase, if one compares the liquid and gel phase images, it can be seen that in both cases substantial spreading of the protein occurs

10 over a wide pI range. This range spans from pI 6.5 to pI 9.5 in both the liquid phase and the gel phase. For more acidic proteins such as ß-actin, it appears that in the liquid phase the protein is more dispersed in the pI dimension than for the corresponding gel separated protein. Both methods provide a reasonably accurate assessment of the pI of the protein of interest. Referring to Table 1, it can be seen

15 that as the Rotofor fraction pH increases, so generally does the pI of identified proteins therein. The pH of fraction 3 measures 4.2 and the proteins identified from this fraction range in pI from 4.09 to 5.7. The pH of fraction 9 was 5.8 and the proteins identified from that fraction ranged from 5.29 to 6.45. The pH of fraction 16 was 7.2 and the pI range of proteins found there ranged from 7.01 to 8.93. The pI accuracy

20 therefore ranges from +/- 0.65 to 1.73 pI units. This is comparable to the carrier ampholyte based gel. It should be remembered that the pI of a given protein may vary significantly due to post-translational modifications such as phosphorylation and glycosylation, as well as to artifactual modifications such as carbamylation and oxidation.

25 **E.   Second Dimension Liquid Separation**

Fraction 16, Figure 4, may be used as an example of the quantification of isolated proteins. For fraction 16, the volume of injection was 160 μL. This means that if the concentration of protein was 201.4 μg/mL then the amount of protein loaded

was 32.2 µg. The chromatogram was integrated using Microcal Origin software and
the total area was determined to be 97.78. The areas of peaks 16E and 16J were 3.68
and 5.41 respectively. Dividing the peak area by the total area gives the fraction of
protein represented by the peak. Therefore, if one assumes 100% protein recovery, the

5   amount of PPIASE (16E, t_R = 5.68) in 16 was (0.0376 * 32.2 µg) 1.21 µg and the
amount of α-enolase (16J, t_R = 7.45) was (0.0553 * 32.3 µg) 1.78 µg. The peak areas
were generated by absorbance of 214 nm light at the amide bonds of the proteins and
so should offer low selectivity thereby allowing for a good measure of the amount of
protein in the peak regardless of the type of protein.

10   Figure 4 shows how the continuous integration of the chromatogram may be
used to estimate the amount of protein isolated in a given peak. The peak area line is
simply converted into mass units from which the observer can measure the change in
the vertical mass axis that occurs over the width of the peak of interest. If one knows
the initial concentration of protein in the cell lysate and the number of cells that were

15   lysed, a quantitative comparison of different cell lysates can be made. This
comparison is important to studying changes in protein expression levels due to some
disease state or pharmacological treatment. In gel work, a technique used for protein
quantification in different samples is to normalize the integrated optical density of the
spot of interest to that of standard proteins whose expression levels are thought to be

20   constant. In this way any experimental variation in spot intensity can be corrected.
This same method is applied to the IEF-NP RP HPLC image to allow for reliable
quantification of proteins of interest such that changes in expression level are
quantitatively observed.

            The assumption in these experiments is 100% protein recovery. One can

25   determine the actual % recovery of protein and the dependence on elution time.
Typical protein recoveries have been shown to range from 70 to 95% in NP RP HPLC
(Wall et al., Anal. Chem., 71:3894 [1999]) and so, with a more likely percent recovery
of 80%, the amount of PPIASE and α-enolase in fraction 16 would be estimated to be
1.0 µg and 1.42 µg, respectively.

F.   Rotofor Fraction Analysis by NP RP HPLC vs. 1-D SDS PAGE

NP RP HPLC provides highly efficient protein separations (*See e.g.*, Chen *et al.*, Rap. Comm. Mass Spec., 12:1994 [1998]; Wall *et al.*, Anal. Chem., 71:3894 [1999]; and Chong *et al.*, Rap. Comm. Mass Spec., 13:1808 [1999]), and is a far

5   easier method to automate as compared to gels in terms of injection, data processing and protein collection. In addition the NP RP HPLC separations provided by the present invention are 70 times faster than the equivalent separation by 1-D SDS-PAGE, which requires 14 hours. In the experiments described above, the NP RP HPLC method has greater resolving power generating 35 bands where the 1-D gel

10   generates only 26 bands. A direct comparison of the two methods, as shown in Figure 7, reveals that the NP RP HPLC bands are much narrower than those of the 1-D SDS PAGE over a similar molecular weight range. Also it is clear that as molecular weight decreases, the 1-D gel band width increases substantially. In NP RP HPLC the opposite trend occurs where the lower molecular weight proteins show improved

15   resolution and sensitivity. This image may appear to show that the NP RP HPLC separation fails with larger proteins as there are few bands in the upper region of the image. However, this is not the case as it is important to remember that the vertical dimension for NP RP HPLC is not protein molecular weight but rather protein hydrophobicity. This is evidenced by the observation of the elution of bovine serum

20   albumin (66 kDa), a relatively hydrophilic protein, half way up an image.

G.   Elution Time Prediction for Known Target Protein

One of the advantages of the 2-D gel is that the vertical coordinate of the gel may be used to estimate the molecular weight of the protein with a +/- 10% error. The position of a protein of interest can therefore be estimated before the protein is

25   identified from the gel. In an attempt to correlate elution time in the methods of the present invention with the mass of the protein, a linear fit to a plot of percent acetonitrile at time of elution (%B) versus the log(MWt)/protein polar ratio was generated. The polar ratio (PR) is the number of polar amino acids divided by the total number of amino acids in the protein and the molecular weight is in kDa. The

proteins used for this plot were four of the standards listed in Figure 1 as well as a sampling of six of the proteins from Table 1 (HSP60, ß-actin, TIM, α-enolase, PPIASE and glyceraldehyde-3-phosphate). The resulting equation (equation 1: %B/100 = 0.079805*(logMWt)/PR + 0.077686, (R = 0.9677, SD = 0.014722, N = 7))

5   is used to predict the elution time of target proteins. For HSP60, ß-actin and α-enolase the experimental elution times were 10.28, 10.15 and 7.25 respectively. The predicted elution times were 10.20, 10.13 and 9.78. In the cases of HSP60 and ß-actin the prediction works well, whereas for α-enolase the prediction is not as good. While not precise, this prediction does give some idea of when a protein will elute such that

10   a given target protein, for which the molecular weight and hydrophobicity are known, can be found more readily.

H.   Protein Identification by Enzymatic Digestion, MALDI-TOF MS and MSFit Database Searching

       The proteins that were identified from a representative sampling of the bands

15   from the IEF-NP RP HPLC separation are listed in Table 1. A sampling of approximately 80 proteins from 12 of the Rotofor fractions were digested and their peptide mass maps successfully obtained by MALDI-TOF MS. Of these 80, 38 different proteins were identified. In this case, identifying roughly 50% of the proteins searched is to be expected as not all the proteins are in the available databases.

20   Similar results were observed for proteins analyzed from 2-D gels of the HEL cell samples. The current table in Swiss-2DPAGE lists 19 protein entries for the HEL cell. Of these 19 proteins, five were identified from the IEF-NP RP HPLC separation. In the gel, these same five proteins were also identified.

       In general, it appears that the gel MSFit results are better than those from the

25   liquid phase. This can be attributed to the fact that the gel proteins were reduced and alkylated with DTE and iodoacetamide respectively prior to the running of the second dimension. This step would help insure that all disulfide bonds are broken and optimal proteolysis is produced. Thus, this derivatization step can be added to the IEF-NP RP HPLC method, by performing the reduction and alkylation step prior to

NP RP HPLC or during cell lysis. Nevertheless, in some cases the IEF-NP RP HPLC digestions surpassed those from the gel in coverage and quality. This is evidenced in Figure 8, which shows a direct comparison of the MALDI-TOF MS for α-enolase as isolated via the IEF-NP RP HPLC method and the gel method. These mass spectra

5   were calibrated externally at first and the mass profiles used to search the Swiss protein database with a mass accuracy of 400 ppm. These searches gave strong hits to α-enolase for both the gel and the liquid protein digests. Each mass spectrum was then recalibrated internally using matched peptide peaks from the initial externally calibrated match. The new peak table was then used to search the same Swiss protein

10   database but with 200 ppm mass accuracy. Figure 8 clearly shows that the digestion from the liquid phase is improved compared to that from the gel. The IEF-NP RP HPLC mass spectrum matches to 60% of the protein sequence whereas that from the gel matches to 49%. Achieving a match to 60% of the sequence of a 47 kDa protein is very unusual for MALDI-TOF MS analysis and represents a significant

15   improvement over gel digests. Although the present invention is not limited to any particular mechanism, the increase in sequence coverage may be due to the fact that the protein is digested in the liquid phase, is relatively pure, and because the peptides are not lost due to being embedded inside the gel piece. Also if one observes the level of methionine oxidation in the peak that matches to T163-179, it is clear that the

20   protein isolated by IEF-NP RP HPLC is far less oxidized than that from the gel.

       Many of the NP RP HPLC chromatograms contain some peaks that are not fully resolved to baseline. This need not be a problem as partially resolved proteins can still be effectively identified using MALDI-TOF MS analysis. In Rotofor fraction 3 there are peaks at 10.15 minutes and 10.25 minutes (See, Table 1). These peaks are

25   only resolved to 50% above the baseline and yet it is clear that the peak eluting at 10.15 minutes is ß-actin and the peak eluting at 10.25 minutes is HSP-60. Note that the predicted elution times for these proteins are 10.13 and 10.20 minutes respectively. As proteins can be identified from partially resolved peaks, faster separations with more rapid gradients are possible. The reproducibility of the pattern of bands can be

30   determined by looking at the retention times for particular proteins as observed from

different Rotofor fractions. ß-actin elutes at 10.15 minutes in both fractions 3 and 9;

α-enolase elutes at 7.25, 7.45 and 7.39 minutes in fractions 12, 16 and 20 respectively;

and HSP-60 elutes at 10.28 and 10.25 minutes in fractions 3 and 4 respectively.

Clearly, with +/- 0.1 minutes variation in the retention times, these separations are

5   quite reproducible from run to run.

        Thus, the methods of the present invention have been shown to provide

advantageous methods for the reproducible separation of large numbers of proteins. In

the human erythroleukemia cell lysate example, the methods are capable of resolving

700 bands with a rapid gradient, and 1000 bands with a longer gradient. There were

10   38 different proteins tentatively identified, by MALDI-TOF MS and MSFit database

searching, after analysis of a fraction of these bands. This compares favorably with

the 19 different proteins that have been identified to date from the 2-D gel. Some of

the proteins found in the human erythroleukemia cell lysate; including α-enolase

(Rasmussen *et al.*, Electrophoresis 19:818 [1998] and Mohammad *et al.*, Enz. Prot.,

15   48:37 [1994]), glyceraldehyde-3-phosphate dehydrogenase (Bini *et al.*, Electrophoresis

18:2832 [1997] and Sirover, Biochim. Biophys. Acta 1432:159 [1999]), NPM (Redner

et al., Blood 87:882 [1996]), CRKL (ten Hoeve *et al.*, Oncogene 8:2469 [1993]), and

heat shock protein (HS27) (Fuqua *et al.*, Cancer Research 49:4126 [1989]), have been

linked to various forms of cancer. NPM and CRKL have been linked specifically to

20   leukemias.

        The proteins identified in one exemplary experiment ranged from 12 kDa up to

75 kDa (although broader ranges are contemplated by the present invention); this range

may include many of the proteins of interest to current research involving protein

profiling, identification and correlation to some disease state or cell treatment. In

25   sharp contrast to 2-D gels, this method is well-suited to automation. Mass

spectrometric methods can be applied, such as ESI-MS and MALDI-TOF MS, to the

detection of whole proteins and protein digests. Most importantly, the methods of the

present invention provide an alternative 2-D protein map to the traditional 2-D gel and

appears to improve results for lower mass proteins and more basic proteins. A key

30   advantage of the liquid 2-D separation is that the end product is a purified protein in

the liquid phase. Also, since the initial protein load can be fifty times that of the gel,
the amount of a target protein that may be isolated by one IEF-NP RP HPLC
separation is potentially fifty times higher than that obtainable from a 2-D gel
separation. Additionally, in the case that the investigator is interested in specific

5   proteins where the pI is known, this method may be used to isolate and identify the
target protein in less than 24 hours, since only the fraction of interest need be analyzed
via the second dimension separation. The gel-based method would require three days
to achieve the same result.

                 I.   Identification of Novel Tumor Antigens
10   There is substantial interest in identifying tumor proteins that are immunogenic.
Autoantibodies to tumor antigens and the antigens themselves represent two types of
cancer markers that can be assayed in patient serum and other biological fluids. IEF-
NP RP HPLC-MS has been implemented for the identification of tumor proteins that
elicit a humoral response in patients with cancers. The identification of proteins that

15   specifically react with sera from cancer patients was demonstrated using this approach.
Solubilized proteins from a tumoral cell line are subjected to IEF-NP RP HPLC-MS.
Individual fractions defined on the basis of pI range are subjected simultaneously to
one-dimensional electrophoresis as well as to HPLC. Sera from cancer patients are
reacted with Western blots of one-dimensional electrophoresis fractions. One band

20   which reacted specifically with sera from lung cancer patients and not from controls
was found to contain both Annexin II and aldoketoreductase. The ability to
subfractionate further proteins contained in this fraction by HPLC led to the
identification of Annexin II as the tumor antigen that elicited a humoral response in
lung cancer patients.

25   J.   Comparative Analysis
               As is clear from the above description, the methods of the present invention
offer the opportunity to compare protein profiles between two or more samples (*e.g.*,
cancer vs. control cells, undifferentiated vs. differentiated cells, treated vs. untreated

cells). In one embodiment of the present invention, the two samples to be compared are run in parallel. The data generated from each of the samples is compared to determine differences in protein expression between the samples. The profile for any given cell type may be used as a standard for determining the identity of future

5   unknown samples. Additionally, one or more proteins of interest in the expression pattern may be further characterized (e.g., to determine its identity). In an alternative embodiment, the proteins from the samples are run simultaneously. In these embodiments, the proteins from each sample are separately labeled so that, during the analysis stage, the protein expression patterns from each sample are distinguished and

10   displayed. The use of selective labeling can also be used to analyze subsets of the total protein population, as desired.

As is clear from the above description, the methods and compositions of the present invention provide a range of novel features that provide improved methods for analyzing protein expression patterns. For example, the present invention provides

15   methods that combine IEF, resulting in pI-focused proteins in liquid phase fractions, with nonporous RP HPLC to produce 2-dimensional liquid phase protein maps. The data generated from such methods may be displayed in novel and useful formats such as viewing a collection of different pI NP RP HPLC chromatograms in one 2-D image displaying the chromatograms in a top view protein band format, not the traditional

20   side view peak format. As shown in Figure 2, the side view peak format is shown to the left and the top view band format is shown to the right. The present invention also provides detergents that are compatible with automated systems employing multi-phase separation and detection steps.

The present invention provides additional characterization steps, including the

25   identification of proteins separated by IEF-NP RP HPLC using enzymatic digestions and mass spectrometric analysis of the resulting peptide mass fingerprints. Proteins may be detected to determine their molecular weights by analyzing the effluent from the HPLC with either off-line collection to a MALDI plate (Perseptive) or on-line analysis using orthogonal extraction time-of-flight. The data generated from such

30   methods may be displayed in novel and useful formats such as using the data from the

MALDI or LCT generated protein molecular weights to generate total ion chromatograms (TIC) that would be virtually identical to the original UV-absorbance chromatograms. The signal of these chromatograms would be based on the number of ions generated from the HPLC effluent of a given group of pI-focused proteins, not by

5   absorption of light. These chromatograms are plotted in the same 2-D top view band format as mentioned above. These methods allow one to fully integrate and deconvolute each of the TIC's generated to display complete mass spectra of each collection of pI-focused proteins. The methods also allow the display of all the integrated TIC's in one 2-D image where the vertical dimension is in terms of protein

10   molecular weight and the horizontal dimension is in terms of protein pI. The protein mass spectra appears as bands as they are also viewed from the top. This image would therefore also contain quantitative information (in the case of the LCT) and so the bands would vary in intensity depending on the amount of protein present.

The liquid phase methods for protein mass mapping would also allow for

15   collection of protein fractions to microtubes such that the proteins could be digested and the peptide mass maps analyzed to determine the identity of said proteins simultaneously. Laser induced fluorescence (LIF) detection schemes are used in conjunction with this method to increase the overall sensitivity by three orders of magnitude. The liquid phase LIF detector provides more sensitive fluorescence

20   detection than in the gel as there would be no gel background fluorescence. This LIF detection method could be used in a number of ways including, but not limited to:

1)   Combining equal amounts of two cell lysates that have each been previously stained with a different fluorescent dye followed by use of a dual fluorescence detector to simultaneously detect the same proteins

25   from two different cell lysates. This would allow for very accurate comparisons of the relative amounts of proteins found for different cell lines or tissues; and

2)   Using a fluorescently tagged antibody to label specific target proteins in a cell lysate such that they can be targeted for thorough analysis without

30   looking at all the other proteins.

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The methods and apparatuses of the present invention also offer an efficient
system for combining with other analysis techniques to obtain a thorough
characterization of a given cell, tissue, or the like. For example, the methods of the
present invention may be used in conjunction with genetic profiling technologies (*e.g.*,

5   gene chip or hybridization based nucleic acid diagnostics) to provide a fuller
understanding of the genes present in a sample, the expression level of the genes, and
the presence of protein (*e.g.*, active protein) associated with the sample.

II)   **Improved Elution Techniques Using Chromatofocusing**

As described above, the present invention provides novel liquid

10   chromatographic methods involving a 2-column 2-D separation of proteins from whole
cell lysates followed by on-line mass mapping with by mass spectrometry (*e.g.*, using
ESI-oaTOF MS as described in detail below). It is a 3-D protein analysis system as
proteins are separated based upon, for example, their isoelectric points (*pI*) in the first
LC dimension.

15   The present invention further provides novel techniques for eluting proteins
from a separation apparatus (*e.g.*, the first phase separation apparatus). For example,
in one embodiment of this technique, the proteins eluted from the first dimension are
"peeled off" from the column according to their pH, either one pH unit or fraction
thereof, at a time—referred to as chromatofocusing (CF). These focused liquid

20   fractions are then separated according to their hydrophobicity and size (or other desired
properties) in the second dimension. Liquid fractions from, for example, NP-RP-
HPLC can be conveniently analyzed directly on-line using mass spectrometry (*e.g.*,
ESI-oaTOF) to obtain their molecular weight and relative abundance, which provides a
third dimension. As a result, a virtual 2-D protein image is created and is analogous

25   to a 2-D gel image. Furthermore, this 2-D protein image includes vital information
such as the *pI*, hydrophobicity, molecular weight, and relative abundance. This
"Protein Peeling" 2-D LC-MS method is a practical alternative to 2-D gels in order to
study protein expression between normal and disease whole cell lysates, for example.

This whole system can be fully automated and integrated into a single unit for rapid proteome analysis, providing a more accurate and less expensive automation technology compared to automation technologies for use with 2-D gels.

5   An exemplary embodiment of the chromatofocusing techniques of the present invention are provided in Example 7. Data from these experiments is shown in Figures 14-16. Figure 14 shows the CF profile of MCF-10A whole cell lysate (pH 7 to 4). Fractions 1 to 3 were further analyzed with NP-RP-HPLC-ESI-oaTOF MS (described in detail below). Figures 15A-C show the NP-RP-HPLC-ESI-oaTOF TIC (total ion count) profile of the three fractions from Figure 14: (A) fraction 1 (pH 6.75 – 6.55); (B) fraction 2 (pH 5.50 – 5.25); and (C) fraction 3 (pH 5.20 – 4.90). By 10 integrating and deconvoluting the TIC profiles with the *MaxEnt1* software (described in detail below), the mass spectra for all three fractions are displayed in a 2-D format as shown in Figure 16. Figure 16 shows the integrated TIC in one 2-D protein map where the vertical column is the molecular weight while the horizontal dimension is the protein *pI* point. This map also contains the relative abundance information 15 whereby the bands vary in intensity (shades of gray) depending on the amount of the protein present.

The data generated by CF-NP-RP-HPLC-ESI-oaTOF MS can be presented as 2-D maps or 2-D images much like the traditional 2-D gel images. For example, in some embodiments, the chromatograms, TICs, integrated and deconvoluted mass 20 spectra are converted into the ASCII format before being plotted vertically, using a 256-step gray scale, such that peaks are represented as darkened bands against a white background. This scale comes in a variety of color formats. Therefore, this 2-D map provides vital information on *pI*, hydrophobicity, molecular weight as well as the 25 relative abundance of separated proteins. This map can also be adjusted by zoom into a specific area of interest, for a more detailed image of all the bands therein. All the information gathered from this 2-D map can be used to examine protein expression in a cell system due to the disease state, pharmaceutical treatment or environmental change. Since the image is automatically digitized, it can be easily stored and the

bands can be hyperlinked to other experimental results or related data. As a result, all the information is available from the original image.

The use of chromatofocusing with the separation, analysis, and display methods of the present invention provide a number of important advantages not previously

5   available. For example, by combining chromatofocusing with a second separation phase (*e.g.*, NP-RP-HPLC) and mass spectrometry analysis, a 2-D liquid phase protein map is generated which is analogous to a 2-D gel. In preferred embodiments, this is a multi-dimensional liquid chromatography (LC) whereby both chromatographic techniques are performed on-line (*i.e.*, in an automated fashion) between two or

10   multiple LC units with a switching valve to deliver fractions from CF to, for example, NP-RP-HPLC. Proteins are "peeled off" the CF column according to their pH, one pH unit or fraction thereof, at a time. This "peeling" feature allows for further focusing of the protein bands at their respective *pI* regions. The protein concentration of each *pI* band is thus enhanced during elution. As with the method described above, buffers

15   can be used that are compatible with each step of the process. For example, in some embodiments, the sample preparation and CF separation involves the use of guanidine-hydrochloride and a nonionic detergent (*e.g.*, n-octyl β-D-glucopyranoside) that is compatible with the NP-RP-HPLC and ESI-oaTOF MS.

III)   Mass Spectroscopic Analysis and 2-D Display Systems and Methods

20   In some preferred embodiments of the present invention, separated proteins are analyzed by mass spectrometry to facilitate the generation of detailed and informative 2-D protein maps. The present invention is not limited by the nature of the mass spectrometry technique utilized for such analysis. For example, techniques that find use with the present invention include, but are not limited to, ion trap mass

25   spectrometry, ion trap/time-of-flight mass spectrometry, quadrupole and triple quadrupole mass spectrometry, Fourier Transform (ICR) mass spectrometry, and magnetic sector mass spectrometry. The following description of mass spectroscopic analysis and 2-D protein display is illustrated with ESI oa TOF mass spectrometry.

Those skilled in the art will appreciate the applicability of other mass spectroscopic techniques to such methods.

In some embodiments of the present invention, ESI oa TOF mass spectrometry is used following two dimensional protein separation to provide an accurate protein separation map. For example, in one embodiments of the present invention, proteins were analyzed from human erythroleukemia (HEL) cells. The human erythroleukemia (HEL) cell line was obtained from the Department of Pediatrics at The University of Michigan. HEL cells were cultured according to the methods described in Example 1. A preparative scale Rotofor (Biorad) was used in the first dimension separation. In this experiment, 20 mg of protein was loaded. The proteins were separated by isoelectric focusing over a 5 hour period with slight modifications to the Rotofor methods described elsewhere herein. The separation temperature was 10°C, and the separation buffer contained 0.5 % n-octyl β-D-glucopyranoside (OG) (Sigma), 6 M urea (ICN), 2 M thiourea (ICN), 2 % β-mercaptoethanol (Biorad) and 2.5 % Biolyte ampholytes, pH 3.5-10 (Biorad).

The procedure used for running the Rotofor (Rotofor Purification System, Biorad) was a modified version of the standard procedure described in the manual from Biorad. The starting power, voltage and current were 12 W, 400 V and 36 mA respectively. The ending power, voltage and current were 12 W, 1000 V and 5 mA respectively. The 20 fractions contained in the Rotofor were collected simultaneously into separate vials using a vacuum source attached by plastic tubing to an array of 20 needles which were punched through a septum. The Rotofor fractions were aliquotted in 400 μL amounts into polypropylene micro-centrifuge tubes and stored at -80°C for further analysis as desired. The pH of the fractions was determined using pH indicator paper (Type CF, Whatman). Fractions from the Rotofor were quantified using a Bradford assay (*See e.g.*, Wall *et al.*, Anal. Chem., 72:1099 [2000]).

For NPS RP HPLC, separations were performed at a flow rate of 0.4 mL per minute on an analytical (3.0 * 33 mm) NPS RP HPLC column containing 1.5 μm C18 (ODSI) non-porous silica beads (Eichrom Technologies). The use of the 3 mm column provided more than sufficient sensitivity with the use of the LCT as well as

reduced solvent consumption. The column was placed in a column heater (Timberline, Boulder CO) and maintained at 65°C. The separations were performed using water/acetonitrile (0.1 % TFA, 0.3% formic acid) gradients. The gradient profile used was as follows: 1) 0 to 20 % acetonitrile (solvent B) in 1 minutes; 2) 20 to 30 % B in

5   2 minutes; 3) 30 to 54 % B in 8 minutes; 4) 54 to 65% B in 1 minute; 5) 65 to 100 % B in 1 minute; 6) 100 % B in 3 minutes; 7) 100 to 5 % B in 1 minute. The effective start point of this profile was one minute into the gradient due to a one-minute dwell time. The acetonitrile was 99.93 +% HPLC grade (Sigma), the TFA was from 1 mL sealed glass ampules (Sigma) and the formic acid was ACS grade (Sigma). The

10   non-ionic detergent used was n-octyl β-D-galactopyranoside (OG) (Sigma). The HPLC instrument used was a Beckman model 127s/166 and the peaks were detected on-line by a commercial ESI oa TOF/MS (LCT, Micromass, Manchester U.K.). In preferred embodiments, a detergent is used throughout the separation and detection steps that is compatible with the steps of RP HPLC and ESI oa TOF/MS (*e.g.*,

15   detergents of the formula n-octyl (SUGAR)pyranoside).

The ESI oa TOF/MS analyses were performed on a Micromass LCT equipped with a reflectron, a 0.5 meter flight tube and a dual micro-channel plate detector. The instrument produced protein mass spectra with a mass resolution of 5000 (FWHM). The flow from the HPLC column eluent was split to the ESI stainless steel capillary at

20   a 1:1 ratio leaving a flow to the mass spectrometer of 0.2 mL/minute. The source temperature was held at 150°C, the desolvation temperature was 400°C, the nebulizer gas (N_2) was left at 50% maximum flow and the desolvation gas was held at 600 L/minute. The capillary voltage was held at +2500 V and the sample cone voltage was held at +45 V. The extraction cone was held at +3 V. The RF voltage was set at

25   1000 V with the first hexapole being biased to a positive DC offset of +7 V and the second hexapole being biased to a negative DC offset of -2 V. The detector voltage was held at 2900 V. Data was acquired for a maximum mass/charge range of 5000 resulting in a pusher cycle time of 90 μs. The data was stored to the ECP at a rate of 1 Hz and then transferred from this data-collecting computer to the main data analysis

30   computer for generation of the data files and TIC.

Software used to analyze the mass spectra was the MaxEnt (version 1) software and Mass Lynx version 3.4 (Micromass). Typical deconvolution was performed with a wide target mass range, 1 Dalton resolution, 0.75 Da peak width and 60% peak height values. All deconvoluted mass spectra from a given TIC were added together to

5   produce one mass spectrum for each TIC. The TIC mass spectra from each of the Rotofor fractions were then input to the 2D mapping software (available from Dr. Stephen J. Parus, University of Michigan, Department of Chemistry, 930 N. University Ave., Ann Arbor, MI 48109-1055).

The 2-D image in Figure 9 shows protein molecular weight in the vertical

10   dimension and protein pI in the horizontal dimension. Individual proteins are represented as bands within the grayscale image. Protein identities were matched to this image by overlaying a virtual map of all proteins previously identified via the NPS RP HPLC separation method described above and digest analysis with MSFit database searching.

15   The experimental mass values were typically within 1 to 3 parts per thousand of the value recorded in the SWISS-PROT database. The pI could be estimated to within 0.01 to 0.5 pI units using intensity profiling as described below. Each vertical lane represents, in band format, all proteins observed via LCT mass spectral detection from the NPS RP HPLC analysis of that particular Rotofor fraction. The NPS RP

20   HPLC separations were performed on from 17 to 60 µg of protein per Rotofor fraction. The bands in the image vary in gray scale intensity according to the intensity of the source molecular weight peaks. This image has been magnified in the intensity dimension by allowing virtual saturation of the signal of the more abundant proteins. The magnification factor is 27X or 53615/2000 (max intensity/magnification intensity).

25   The intensity has a linear dynamic range of at least 3 orders of magnitude. Some of the same protein patterns can be seen in both the liquid phase separation and a 2D gel image from Swiss-Prot (http://expasy.cbr.nrc.ca/ch2dothergifs/publi/elc.gif). Five of the nineteen proteins identified in the 2D gel image also were found in the liquid phase separation. When comparing these images it must be kept in mind that the mass

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scale is linear from the liquid phase separation and logarithmic in the gel phase separation.

The pI of proteins isolated in the 3D liquid separation method can be estimated by observing the intensity of a given protein peak over a range of pI fractions. As a

5 protein may spread anywhere from 2 to 6 pI fractions due to diffusion and basic cathodic drift, it should be most abundant in that fraction that is closest to its own pI. This can be observed in the zoom image of Figure 10 (*See also*, zoom image of Figure 13). Using this approach, the pI of alpha-enolase is estimated to be 7.0 (database value of 7.01), and the pI of glyceraldehyde 3-PO_4 dehydrogenase is estimated to be

10 8.0 (database value of 8.57). This acidic shift may be due to a post-translational modification such as phosphorylation or glycosylation.

The protein molecular weights were determined by MaxEnt deconvolution of multiply charged protein umbrella mass spectra that were obtained by combining anywhere from 10 to 60 seconds of data from the initial total ion chromatogram (TIC).

15 The umbrella for beta and gamma actin is shown in Figure11A, each form of actin being labeled with the charge state. Figure 11B shows the resulting molecular weight mass spectrum for actin where the two forms of actin are separated. Note that the two forms of actin are clearly resolved from one another unlike in gel images where the actin spot always represents the co-migration of beta and gamma actin. A useful

20 feature of the liquid phase method of the present invention is the capability of the high resolution mass spectrometry to quantitate which allows the observer to record relative levels of each form of a given protein. Consequently, it is contemplated that one cam determine the relative abundances of the phosphorylated and non-phosphorylated forms of a given protein. In addition, post-translational modifications such as

25 phosphorylation can be found by searching the data for intervals of some integer value times 80 Da.

Figure 12 shows the traditional peak view format of one of the Rotofor fraction's combined molecular weight mass spectra. All proteins were deconvoluted and then added together into one mass spectrum. There are 44 unique protein

30 molecular weights observed in this mass spectrum. Assuming similar numbers of

unique masses in all 15 of the Rotofor fractions analyzed herein, and accounting for longitudinal diffusion between fractions, it is estimated that approximately 220 unique protein masses in the image from a pI of 4.1 to a pI of 8.75. The Rotofor produces 20 fractions, though only 15 were analyzed in this work, so that around 300 unique

5   masses should be observed in the full analysis of all Rotofor fractions. It is contemplated that lower level proteins not obtained in the above experiment can be obtained using improved HPLC gradients, 53 mm long columns and more detailed MaxEnt analyses. Using such methods, it is contemplated that the number of unique masses will be around 750.

10   As shown in the above experiments, the 2D protein image from the IEF-NPS RP HPLC-ESI oa TOF/MS separation of the human erythroleukemia cell lysate provides high mass resolution and high accuracy imaging of the proteins. The mass resolution allows the image to show very different forms of the same protein that have small differences in mass. With a mass resolution of 5000 Da, a 50000 Da protein can

15   be resolved from a 50010 Da protein. Clearly, single phosphorylations on entire proteins can be observed with this level of resolution. Quantitative comparison between 2-D images can be achieved by spiking samples with known amounts of standard proteins and normalizing images through landmark proteins. Thus, the observer can detect significant abundance changes in the protein profiles of different

20   samples. The differences can then be targeted for more detailed analysis. For example, protein bands on the image can be hyper-linked to other experimental results, obtained via analysis of that band, such as peptide mass fingerprints and MSFit search results. Thus all information obtained about a given 2-D image, including detailed mass spectra, data analyses and complementary experiments (immuno-affinity, peptide

25   sequencing) can be accessed from the original image.

Having identified and characterized the proteins that have changed in abundance due to some disease state or drug treatment, it is possible to identify biomarkers for disease states as well as drug targets for pharmaceutical agents and monitor the presence of, or change in, such markers in a particular biological sample

30   (e.g., tissue samples with and without exposure to a candidate drug). Indeed, drug

screening and diagnostic techniques can be automated using the systems and methods of the present invention, wherein cells (*e.g.*, experimental and control cells) are cultured, treated, and lysed using robotics and wherein the lysate is fed into the automated separation and analysis systems of the present invention.

5   As is clear from the above description, the methods and systems of the present invention provide a range of novel features that provide improved methods for analyzing protein expression patterns. For example, the present invention provides a combination of IEF, resulting in pI-focused proteins in liquid phase fractions, with nonporous RP HPLC and ESI oa TOF/MS to produce a 2-dimensional liquid phase

10   protein map image analogous to that of a 2-D gel. These methods allow the identification of proteins separated by IEF-NPS RP HPLC using enzymatic digestions and mass spectrometric analysis of the resulting peptide mass fingerprints and correlation of this data with the pI and molecular of the protein found via the whole protein 3-D separation method. In some improved display embodiments of the present

15   invention, one can view a collection of different IEF-NPS RP HPLC-ESI oa TOF/MS chromatograms in one 2-D image displaying the mass spectra in a top view protein band format, not the traditional side view peak format. The methods also allow the detection of proteins and determination of their molecular weights by analyzing the eluent from the HPLC with computational (*e.g.*, on-line) analysis using ESI oa

20   TOF/MS.

          The IEF-NPS RP HPLC-ESI oa TOF/MS method also allows one to fully integrate and deconvolute each of the TIC's generated to display complete mass spectra of each collection of pI-focused proteins. The method also allows the display of all the integrated TIC's in one 2-D image where the vertical dimension is in terms

25   of protein molecular weight and the horizontal dimension is in terms of protein pI. In such displays, the protein mass spectra appear as bands as they will also be viewed from the top. This image would therefore also contain relative quantitative information wherein the bands vary in intensity depending on the amount of protein present. The use of liquid phase separation techniques with the method allows for

30   collection of protein fractions to micro-tubes or 96-well plates such that the proteins

could be digested and the peptide mass maps analyzed to determine the identity of said proteins simultaneously.

IV)   Automated 3D HPLC/MC Methods for Rapid Protein Characterization

In some embodiments, the present invention provides an automated system for

5   the separation and identification of protein samples based on multiple physical properties. Accordingly, in some embodiments, the protein separation and analysis techniques described in the preceding sections are automated into one integrated, on-line system. Protein samples are separated in a first phase and a second orthogonal phase, followed by mass spectroscopy analysis. In preferred embodiments, all of the

10   steps are automated and coordinated through an automated sample handler and a centralized control network.

Accordingly, in some embodiments, the entire separation and characterization process is controlled through one centralized control network. The network is integrated with all of the apparatus and software used for the automated process. In

15   some preferred embodiments, the centralized control network includes a computer system. The use of a centralized control network allows for the entire separation and characterization process to be controlled from one computer terminal by one operator. The network directs sample through the appropriate separation phases. The network then controls the transfer of protein information to analysis software. The analysis

20   software is integrated into the network and can be programmed to generate a customized report based on the information required by the user.

A.   Protein Separation

As described above, the present invention provides methods for the separation of protein samples in two phases. In preferred embodiments, the methods are

25   orthogonal, and thus allow for the generation of a two-dimensional map. In some preferred embodiments, the present invention further provides methods of automating the two phase separation.

1.   Separation in a First Phase

The automated separation methods of the present invention may be used on any suitable protein sample. As discussed above, in some embodiments, the sample is solubilized in a buffer comprising a compound of the formula n-octyl SUGAR

5   pyranoside (*e.g.*, including, but not limited to, n-octyl β-D-glucopyransoside and n-octyl β-D-galactopyransoside).

The first dimension of the automated separation process separates proteins based on a first physical property. For example, in some embodiments of the present invention proteins are separated by charge (*e.g.*, ion exchange chromatography). In

10   some preferred embodiments, cation exchange chromatography is used to separate positive proteins and anion exchange chromatography is used to separate negatively charged proteins. However, the first dimension may employ any number of separation techniques including, but not limited to, ion exclusion, isoelectric focusing, normal/reversed phase partition, size exclusion, ligand exchange, liquid/gel phase

15   isoelectric focusing, and adsorption chromatography.

In some preferred embodiments, the first separation phase is conducted in the liquid phase. In some embodiments, the first phase is ion exchange. In such embodiments, it is preferred that samples are de-salted prior to the second separation phase. In some embodiments, desalting is performed on an automated solid phase

20   extraction (SPE) system. In some embodiments, both the ion exchange and the desalting are performed on the same automated SPE system. In other embodiments, the ion exchange is performed on a column and the eluate is directed into the automated SPE system.

In some embodiments, if proteins are present in small amounts, samples can be

25   loaded onto the SPE columns multiple times in order to obtain a sufficient amount for analysis. Thus, the present invention has the added advantage of allowing the identification of proteins with a low level of expression.

2.   Automated Sample Handling

As described in the preceding section, in preferred embodiments, samples are processed using an automated sample handling system. The present invention is not limited to any one automated sample handling system. However, in some preferred

5   embodiments, an on-line automated, SPE system is utilized (e.g., including, but not limited to, the Prospekt automated SPE system; Spark Holland Instrumenten, The Netherlands). The advantage of on-line SPE is the direct elution of the extract from the SPE cartridge into the second phase (e.g., LC system) by the LC mobile phase. Several laborious handling steps are thus omitted, making on-line SPE much more

10   efficient and providing superior analytical results. The superior analytical performance of on-line SPE is derived from the elimination of eluate collection, evaporation, reconstitution and injection, thus eliminating several major error sources. In addition, on-line elution transfers 100% of the purified analytes from the extraction cartridge into the LC (e.g., HPLC). This provides maximum precision and sensitivity, as well

15   as reduced costs, thus saving solvents, glassware, and labor time. In addition, samples and SPE cartridges are processed in a completely closed system making sample tracking easy and protecting samples against light and air. It also protects the operator from contact with hazardous samples or solvents. Furthermore, less handling means fewer failures and high pressure solvent control for SPE makes the process

20   independent of cartridge back pressure.

3.   Separation in a Second Phase

In some preferred embodiments, following the first separation phase, products of the separation step are fed directly into a second liquid phase separation step. The second dimension separates proteins based on a second physical property (i.e., a

25   different property than the first physical property) and is preferably conducted in the liquid phase (e.g., liquid-phase size exclusion). For example, in some embodiments of the present invention, proteins are separated by hydrophobicity using non-porous reversed phase HPLC (See e.g., Liang et al., Rap. Comm. Mass Spec., 10:1219 [1996];

Griffin *et al.*, Rap. Comm. Mass Spec., 9:1546 [1995]; Opiteck *et al.*, Anal. Biochem. 258:344 [1998]; Nilsson *et al.*, Rap. Comm. Mass Spec., 11:610 [1997]; Chen *et al.*, Rap. Comm. Mass Spec., 12:1994 [1998]; Wall *et al.*, Anal. Chem., 71:3894 [1999]; Chong *et al.*, Rap. Comm. Mass Spec., 13:1808 [1999]).

5   This method provides for exceptionally fast and reproducible high-resolution separations of proteins according to their hydrophobicity and molecular weight. The non-porous (NP) silica packing material used in these reverse phase (RP) separations eliminates problems associated with porosity and low recovery of larger proteins, as well as reducing analysis times by as much as one third.

10   In preferred embodiments, an automated on-line sample handling system utilized in the present invention fully integrates the second separation phase with the first separation step. The sample flows directly from the first phase (*e.g.*, ion exchange) through a desalting step (*e.g.*, SPE) to the second phase (*e.g.*, NP-RP HPLC). In preferred embodiments (*e.g.*, those utilizing the Prospekt system) the

15   HPLC column is integrated into the automated sample handling system. For example, a multi valve system can be utilized where valve-switching is used to bring the extraction cartridge into the HPLC system. In some embodiments, a sample is passed through the second phase separation step (*e.g.*, NP-RP HPLC) greater than one time (*e.g.*, twice) in order to improve selectivity and resolution. For example, in some

20   embodiments, two different NP-RP-HPLC columns are utilized in tandem. The automation of protein separation increases efficiency and speed as well as decreases sample loss or potential contamination that may occur through handling.

B.   Protein Identification by Mass Spectroscopy

Following separation in the first and second phase, the automated sample

25   handling system transfers samples to the mass spectroscopy step. The present invention is not limited to any one mass spectroscopy technique. Indeed, a variety of techniques are contemplated. For example, techniques that find use with the present invention include, but are not limited to, ion trap mass spectrometry, ion trap/time-of-

flight mass spectrometry, quadrupole and triple quadrupole mass spectrometry, Fourier Transform (ICR) mass spectrometry, and magnetic sector mass spectrometry. In preferred embodiments, the MS analysis is automated and is performed on-line. In some embodiments, the eluent from the second separation phase is split into two

5   fractions. A fraction of the effluent is used to determine molecular weight by either MALDI-TOF-MS or ESI oa TOF (LCT, Micromass) (*See e.g.,* U.S. Pat. No. 6,002,127). The remainder of the eluent is used to determine the identity of the proteins via digestion of the proteins and analysis of the peptide mass map fingerprints by either MALDI-TOF-MS or ESI oa TOF. The molecular weight 2-D protein map is

10   matched to the appropriate digest fingerprint by correlating the molecular weight total ion chromatograms (TIC's) with the UV-chromatograms and by calculation of the various delay times involved. The UV-chromatograms are automatically labeled with the digest fingerprint fraction number. The resulting molecular weight and digest mass fingerprint data can then be used to search for the protein identity via web-based

15   programs like MSFit (UCSF).

A detailed discussion of the use of 3-D maps generated by the automated separation process of the present invention to identify and characterize proteins is provided in the above sections. In some embodiments, the present invention provides a 3-D map in which the first dimension represents a first physical property (*e.g.,*

20   charge or isoelectric point), the second dimension represents a second physical property (*e.g.,* hydrophobicity or molecular weight), and the third dimension represents the molecular weight and relative abundance of proteins present in the sample. In some embodiments, the data from the 3-D protein map is used to search protein data bases in order to determine the identity of the proteins.

25   In some embodiments of the present invention, sample analysis is automated and integrated with the centralized control network. For example, mass spectroscopy data is transferred to an integrated computer system containing software for the generation of 3-D protein maps. The integrated computer system is also capable of searching databases and generating a report. The report is provided to the operator in

30   a format that is customized to the particular application. For example, if an

experiment was designed to identify unknown components of a solution, the report identifies components of the 3-D map as particular proteins. Conversely, if an experiment is designed to compare the protein expression profiles of two samples, the report may identify proteins that are present in one sample and absent in another or are present at different abundances between the two samples.

C.   Automated Protein Separation and Characterization in Practice

Illustrative Example 8 describes one particular embodiment of the present invention where an automated on-line Prospekt system was used to separate a protein sample based on charge and hydrophobicity. Siberian Permafrost whole cell lysate was first separated using a mini MonoQ anion exchange column. A graph of the Mini Q column eluent is shown in Figure 17. Fractions (1 minute each) from the anion exchange column gradient were fed directly into the second step using the automated Prospekt system. The Prospekt then trapped the fractions on 10 C4 SPE cartridges. Each cartridge was washed with the reverse-phase HPLC starting buffer to remove residual salt. The Prospekt system integrates the HPLC and SPE steps with a multi valve switching system. Following the wash step, the eluent from the SPE cartridge was directly transferred to the NP-RP HPLC column.

The fractions were separated using a tandem column method. A gradient was applied to the HPLC column. The HPLC column was then switched back to the initial buffer and allowed to equilibrate. The eluent from the first gradient is then passed through a second (different) HPLC column. The use of a second tandem column increases resolution and selectivity. This step is repeated for each of the SPE cartridges (each representing one anion exchange fraction).

Following separation by NP-RP-HPLC, protein fractions were analyzed online by MS to determine their molecular weight and abundance. The eluent from the column was split into two fractions. One fraction is digested enzymatically before MS. Both the digested and non-digested sample were analyzed by ESI oa TOF TIC (total ion count) mass spectroscopy. Total ion count profiles are shown in Figures 18A and 18B.

EXPERIMENTAL

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

5

EXAMPLE 1

HEL Cell Sample Preparation

The human erythroleukemia (HEL) cell line was obtained from the Department of Pediatrics at The University of Michigan. HEL cells were cultured (7% CO_2, 37 °C) in RPMI-1640 medium (Gibco) containing 4 mM glutamine, 2 mM pyruvate, 10

10   % fetal bovine serum (Gibco), penicillin (100 units per mL), streptomycin (100 units per mL) and 250 mg of hygromycin (Sigma). The HEL cell pellets were washed in sterile PBS, and then stored at –80 °C. The cell pellets were then re-suspended in 0.1% n-octyl ß-D-galactopyranoside (OG) (Sigma) and 8 M urea (Sigma) and vortexed for 2 minutes to effect cell disruption and protein solubilization. The whole cell

15   protein extract was then diluted to 55 mL with the Rotofor buffer and introduced into the Rotofor separation chamber (Biorad).

EXAMPLE 2

1-D Gel and SDS PAGE Separation

HEL cell proteins, resolved by Rotofor separation into discrete pI ranges, were

20   further resolved according to their apparent molecular weight by SDS-PAGE. This procedure takes approximately 14 hours to complete. Samples of rotofor fractions were suspended in an equal volume of sample buffer (125 mM Tris (pH 6.8) containing 1% SDS, 10% glycerol, 1% dithiothreitol and bromophenol blue) and boiled for 5 min. They were then loaded onto 10% acrylamide gels. The samples

25   were electrophoresed at 40 volts until the dye front reached the opposite end of the gel. The resolved proteins were visualized by silver staining. The gels were fixed overnight in 50% ethanol containing 5% glacial acetic acid, then washed successively (for 2 hours each) in 25% ethanol containing 5% glacial acetic acid, 5% glacial acetic

acid, and 1% glacial acetic acid. The gels were impregnated with 0.2% silver nitrate for 25 min. and were developed in 3% sodium carbonate containing 0.4% formaldehyde for 10 min. Color development was terminated by impregnating the gels with 1% glacial acetic acid, after which the gels were digitized.

EXAMPLE 3

2-D PAGE

5

In order to prepare protein extracts from the HEL cells, the harvested cell pellets were lysed by addition of three volumes of solubilization buffer consisting of 8 M urea, 2% NP-40, 2% carrier ampholytes (pH 3.5 to 10), 2% ß-mercaptoethanol and

10   10 mM PMSF, after which the buffer containing the cell extracts was transferred into microcentrifuge tubes and stored at –80 ° C until use.

Extracts of the cultured HEL cells were separated in two dimensions as previously described by Chen *et al.* (Chen *et al.*, Rap. Comm. Mass Spec. 13:1907 [1999]) with some modifications as described below. Subsequent to cellular lysis in

15   solubilization buffer, the cell lysates from approximately 2.5×10^6 cells were applied to isoelectric focusing gels. Isoelectric focusing was conducted using pH 3.5 to 10 carrier ampholytes (Biorad) at 700 V for 16 h, followed by 1000 V for an additional 2 hours. The first dimension tube gel was soaked in a solution of 2 mg/mL of dithioerythritol (DTE) for 10 minutes, and then soaked in a solution of 20 mg/mL of

20   iodoacetamide (Sigma) for 10 minutes, both at room temperature. The first-dimension tube gel was loaded onto a cassette containing the second dimension gel, after equilibration in second-dimension sample buffer (125 mM Tris (pH 6.8), containing 10% glycerol, 2% SDS, 1% dithioerythritol and bromophenol blue). For the second-dimension separation, an acrylamide gradient of 11.5% to 14% was used, and the

25   samples were electrophoresed until the dye front reached the opposite end of the gel. The separated proteins were transferred to an Immobilon-P PVDF membrane. Protein patterns in some gels were visualized by silver staining or by Coomassie blue staining, and on Immobilon-P membranes by Coomassie blue staining of the membranes.

EXAMPLE 4

Rotofor Isoelectric Focusing

A preparative scale Rotofor (Biorad) was used in the first dimension separation. This device separated the proteins in liquid phase according to their pI, and is capable

5   of being loaded with up to a gram of protein, with the total buffer volume being 55 mL. Alternatively, for analysis of smaller quantities of protein, a mini-Rotofor with a reduced volume can be used. These proteins were separated by isoelectric focusing over a 5 hour period where the separation temperature was 10 °C and the separation buffer contained 0.1 % n-octyl ß-D-galactopyranoside (OG) (Sigma), 8 M urea (ICN),

10   2 % ß-mercaptoethanol (Biorad) and 2.5 % Biolyte ampholytes, pH 3.5-10 (Biorad). The procedure used for running the Rotofor (Rotofor Purification System, Biorad) was of the standard procedure described in the manual from Biorad as modified herein. The 20 fractions contained in the Rotofor were collected simultaneously, into separate vials using a vacuum source attached by plastic tubing to an array of 20 needles,

15   which were punched through a septum. The Rotofor fractions were aliquotted into 400 µL amounts in polypropylene microcentrifuge tubes and could be stored at –80 °C for further analysis if necessary. An advantage of gel methods is the ability to store proteins stably in gels at 4 °C for further use. The concentration of protein in each fraction was determined via the Biorad Bradford based protein assay. The pH of the

20   fractions was determined using pH indicator paper (Type CF, Whatman).

EXAMPLE 5

NP RP HPLC

Separations were performed at a flow rate of 1.0 mL/minute on an analytical (4.6 * 14 mm) NP RP HPLC column containing 1.5 µm C18 (ODSI) non-porous silica

25   beads (Micra Scientific Inc.). The column was placed in a Timberline column heater and maintained at 65 °C. The separations were performed using water/acetonitrile (0.1% TFA, 0.05 % OG) gradients. The gradient profile used was as follows: 1) 0 to 25% acetonitrile (solvent B) in 2 minutes; 2) 25 to 35% B in 2 minutes; 3) 35 to 45% B in 5 minutes; 4) 45 to 65% B in 1 minute; 5) 65 to 100% B in 1 minute; 6) 100%

B in 3 minutes; 7) 100 to 5% B in 1 minute. The start point of this profile was one

minute into the gradient due to a one-minute dwell time. The acetonitrile was

99.93+% HPLC grade (Sigma) and the TFA were from 1 mL sealed glass ampules

(Sigma). The non-ionic detergent used was n-octyl ß-D-galactopyranoside (OG)

5   (Sigma). The HPLC instrument used was a Beckman model 127s/166. Peaks were

detected by absorbance of radiation at 214 nm in a 15 µL analytical flow cell.

Protein standards (Sigma) used as MW protein markers and for correlation of

retention time, molecular weight and hydrophobicity were bovine serum albumin (66

kDa), carbonic anhydrase (29 kDa), ovalbumin (45 kDa), lysozyme (14.4 kDa), trypsin

10   inhibitor (20 kDa) and α-lactalbumin (14.2 kDa).

EXAMPLE 6

MALDI-TOF MS of NP RP HPLC Isolated Proteins

The MALDI-TOF MS analyses were performed on a Perseptive Voyager

Biospectrometry Workstation equipped with delayed extraction technology, a one-

15   meter flight tube and a high current detector. The N_2 laser provided light at 337 nm

for laser desorption and ionization. MALDI-TOF MS was used to determine masses

of peptides from protein digests using a modified (described herein) version of the two

layer dried droplet method of Dai et al. (Dai et al., Anal. Chem., 71:1087 [1999]).

The MALDI matrix α-cyano-4-hydroxy-cinnamic acid (α-CHCA) (Sigma Chemical

20   Corp., St Louis, MO, USA) was prepared in a saturated solution of acetone (1% TFA).

This solution was diluted 8-fold in the same acetone solution (1% TFA) and then

added to the sample droplet in a 1:2 ratio (v:v). The mixed droplet was then allowed

to air dry on the MALDI plate prior to introduction into the MALDI TOF instrument

for molecular weight analyses.

25   The proteins were collected into 1.5 mL polypropylene micro-tubes containing

20 µL of 0.8 % OG in 50 % ethanol. In preparation for enzymatic digestion the

acetonitrile was removed via speedvac at 45 °C for 30 minutes. A solution of 200 mM

NH_4HCO_3 (ICN) / 1mM ß-mercaptoethanol was then added in a 1 to 2 ratio to the

remaining solution in the tubes, resulting in a solution of 50 to 100 mM NH_4HCO_3

with a total volume of approximately 150 μL. Subsequently 0.25 μg of enzyme was

added to this solution and then the mixture was vortexed and placed in a 37 °C warm

room for 24 hours. The enzymes used were either trypsin (Promega, TPCK treated),

which cleaves at the carboxy side of the arginine and lysine residues, or Glu-C

5   (Promega), which in 50 - 100 mM NH₄HCO₃ solution cleaves at the carboxy side of

the glutamic acid residues.

The digest solutions were typically 100 μL in volume and 30 to 50 μL of this

solution was desalted and concentrated to a final volume of 5 μL using Zip-Tips

(Millipore) with 2 μL C18 resin beds. The purified peptide solution was then used to

10   spot onto the MALDI plate for subsequent MALDI-TOF MS analysis. All spectra

were obtained with 128 averages and internally or externally calibrated using the

PerSeptive standard peptide mixture containing angiotensin I, ACTH(1-17), ACTH(18-

39) and ACTH(7-38) (PerSeptive Biosystems).

These digests were then used to aid in the identification of the proteins by

15   MALDI-TOF MS analysis and MSFit database searching (Wall *et al.*, Anal. Chem.,

71:3894 [1999]). The peptide mass maps were searched against the Swiss and NCBInr

protein databases using MSFit allowing for 2 missed cleavages. The molecular weight

ranged from 5 kDa to 70 kDa and the pI ranged over the full pI range. Externally

calibrated peptide masses were searched with 400 ppm mass accuracy and internally

20   calibrated peptide masses were searched with 200 ppm mass accuracy.

EXAMPLE 7

Chromatofocusing

In one exemplary embodiment of the chromatic focusing techniques of the

present invention, proteins are extracted from cells using chemical lysing procedure.

25   The lysis buffer consists of 6M guanidine-hydrochloride, 20 mM n-octyl β-D-

glucopyranoside and 50 mM Tris. Cells are vortexed rigorously and kept overnight at

− 20 °C. They are subsequently centrifuged at 17,000 rpm for 20 min. The

supernatant is removed from the cell debris and re-centrifuged at high speed to further

remove any particulate. For the best reproducible results, lysate is best used within 48

hrs. Buffers for this CF are (A) Imidazole-HAC, 0.1% guanidine-hydrochloride, 0.05% n-octyl β-D-glucopyranoside, pH 7.2, and (B) Polybuffer 74 (diluted 1:10), 0.1% guanidine-hydrochloride, 0.05% n-octyl β-D-glucopyranoside, pH 4. The CF column in this example is Mono P HR 5/20 (Amersham Pharmacia, Uppsala, Sweden)

5   with a flowrate of 1 mL/min at room temperature. Prior to injection lysate is equilibrated with buffer A with a loading time of 20 min. The sample loadability for this CF column is 10 mg of protein. The separation profile is monitored at 280 nm while the pH gradient is monitored using a pH flowcell meter, also from Amersham Pharmacia.

10   The CF column is equilibrated with buffer A to define the upper pH range (7 in this case) of the pH gradient. The second "focusing" buffer B is then applied to elute bound proteins, in the order of their isoelectric (pI) points. The pH of buffer B is 4, which defines the lower limit of the pH gradient. The pH gradient is formed as the eluting buffer B titrates the buffering groups on the ion-exchanger.

15   The pI-focused liquid fractions from CF are analyzed in the second dimension using NP-RP-HPLC. Non-porous RP-HPLC columns (Eichrom Technologies, Darien, IL, USA) are used as the second orthogonal separation dimension after CF in order to obtain a 2-D protein map that is capable of competing with 2-D gel. These columns are excellent for protein separation due to their high protein recovery, speed and

20   efficiency. To achieve optimal protein separation, the columns should be kept at a high temperature (*e.g.*, 60 °C). This elevated temperature also improves selectivity. Selectivity as well as resolution can also be enhanced by using multiple NP columns in series. RP-HPLC columns packed with non-porous silica beads (Eichrom Technologies) such as ODS1, 2 and 3 are all well suited for these tasks.

25   Proteins that elute from NP-RP-HPLC separation can be directly analyzed by MS to determine their molecular weight, identity and relative abundance. In this case the eluted proteins are sized simultaneously by ESI-oaTOF MS (LCT, Micromass, Manchester, UK). The other part of the eluted proteins from the split valve can be collected using a fraction collector for enzymatic digestion to obtain peptide maps with

30   a MALDI-TOF MS, ESI-QIT-reTOF MS, or ESI-oaTOF MS (LCT). Information

such as the molecular weight, *pI* and peptide map of a protein can then be entered into a web-based protein database program such as MS-Fit (*e.g.*, http://prospector.ucsf.edu) for protein identification.

Example 8

5
Automated 3-D IE NP-RP-HPLC-ESI-oa TOF MS

This example describes an automated system for protein separation and identification based on charge, hydrophobicity, and mass. Protein samples are separated based on charge using an ion exchange (IE) column. Protein fractions are then trapped on a solid phase extraction (SPE) column for desalting using an

10   automated Prospekt system. The Prospeckt system then directs the protein fractions to a nonporous-reverse phase HPLC column (NP-RP-HPLC). The samples are then identified using ESI oa TOF mass spectroscopy.

A.   Protein Separation and Trapping by SPE

Siberian Permafrost whole cell lysate of sample 23-9-25 (obtained from Jim

15   Tiendje, Department of Microbial Ecology, Michigan State University) was lysed using a chemical lysis procedure. The lysis buffer contained 6M guanidine-HCL, 20 mM n-octyl β-D-glucopyransoside and 50 mM Tris. The cells were vortexed vigorously and stored overnight at 0°C. The cells were then centrifuged at 17,000 rpm for 20 minutes. The supernatant was removed from the cellular material and then mixed 1:1

20   with an equilibration buffer for IE (10 mM KH_2PO_4, 5%MeOH, 0.1 % n-octyl β-D glucopyranoside, pH 8). The sample was then injected into a Mini Q anion exchange column (Amersham Pharmacia, Uppsala, Sweden) with a flow rate of 1 ml/min at 27°C. Equilibration buffer was run through the column for 3 minutes, followed by a 0% to 100% gradient of buffer B (10 mM KH_2PO_4, 5%MeOH, 0.1 % n-octyl β-D

25   glucopyranoside, 1M NaCl, pH 7) in 15 minutes. A graph of the Mini Q column eluent is shown in Figure 17.

Fractions (1 minute each) are each collected on a separate solid phase extraction (SPE) cartridge by directing the eluent from the IE through 10 C4 SPE

cartridges. A Prospekt on-line automated SPE system (Spark Holland Instrumenten, The Netherlands) was utilized for the SPE, HPLC, and MS phases.

B.   Protein Purification and Separation by NP-RP-HPLC

The initial mobile phase buffer for the RP analysis was 5 % buffer B (0.1%

5   TFA in ACN) in buffer A (0.1 % TFA in H_2O). This solution was directed through the SPE cartridge until all the residual salt from the anion exchange mobile phase was removed. The eluent from the SPE cartridge was next directed by the Prospekt system directly to a HPLC for the second orthogonal separation phase.

Non Porous-RP columns (Eichrom Technologies, Darien, IL) were used as the

10   second separation phase. A tandem column method was employed. ODSIIIE and ODSI NP RP HPLC columns (Eichrom Technoligies, Darien, IL) contained 1.5 μm C18 (ODSI) non-porous silica beads. Column dimentions were 4.6 * 33 mm (ODSIIIE) and 4.6 * 14 mm (ODSI). The columns were maintained at 60°C to improve selectivity. A flow rate of 0.5 mL/min at a pressureof 5000 psi was

15   maintained. The columns were loaded, equilibrated in the initial buffer, and the gradient was started. A gradient of buffer B (0.1% TFA in ACN) was performed as follows: 5% B for 1.5 min, 5% B to 20% B in 2 min, 20% B to 35% B in 5 min, 35% B to 60% B in 15 min, 60% B to 100% B in 5 minutes. The eluent from the first HPLC column (ODSI) was directed into the second HPLC column (ODSIIIE).

20   Following the gradient, the initial mobile phase buffer was run through the RP column until a stable baseline is realized. The HPLC step was repeated for each of the SPE columns (each of which contained a 1 minute fraction from the anion exchange column).

C.   Protein Identification by Mass Spectroscopy

25   Following separation by NP-RP-HPLC, protein fractions were analyzed online by MS to determine their molecular weight and abundance. Samples were analyzed by ESI oa TOF TIC (total ion count) mass spectroscopy. Mass spectroscopy conditions

were as follows: capillary 2900V, sample cone 45V, extraction cone 3V, RF lens

1000V, desolution temp or 350°C, and source temp of 120°C.

Results of the ESI oa TOF TIC analysis are shown in Figures 18A and B.

Figure 18A shows the total ion profile of the fraction collected from 3 to 4 of the

5   MiniQ column; figure 18B shows the total ion profile of the fraction collected from 7

to 8 minutes.

All publications and patents mentioned in the above specification are herein

incorporated by reference. Various modifications and variations of the described

method and system of the invention will be apparent to those skilled in the art without

10   departing from the scope and spirit of the invention. Although the invention has been

described in connection with specific preferred embodiments, it should be understood

that the invention as claimed should not be unduly limited to such specific

embodiments. Indeed, various modifications of the described modes for carrying out

the invention which are obvious to those skilled in the art are intended to be within the

15   scope of the following claims.

CLAIMS

We Claim:

1.   A method for characterizing proteins comprising:

        a)   providing:

5

            i)   a sample comprising a plurality of proteins;

            ii)   a first separating apparatus that separates proteins based on a first physical property;

            iii)   a second separating apparatus that separates proteins based on a second physical property; and

10

            iv)   a mass spectrometry apparatus;

        b)   treating said sample with said first separating apparatus to produce a first separated protein sample;

        c)   treating at least a portion of said first separated protein sample with said second separating apparatus to produce a second separated protein

15   sample; and

        d)   directly feeding said second separated protein sample from said second separating apparatus to said mass spectrometry apparatus; and

        e)   mass spectrally analyzing at least a portion of said second separated protein sample with said mass spectrometry apparatus to characterize

20   protein mass.

2.   The method of Claim 1, wherein said sample comprises a cell lysate.

3.   The method of Claim 1, wherein said first physical property is protein charge.

4.   The method of Claim 1, wherein said first separating apparatus

25   comprises an isoelectric focusing apparatus.

5.   The method of Claim 1, wherein said first separating apparatus
comprises a liquid phase separating apparatus.

6.   The method of Claim 1, wherein said second separating apparatus
comprises a reverse phase HPLC apparatus.

5   7.   The method of Claim 6, wherein said reverse phase HPLC comprises
non-porous reverse phase HPLC.

8.   The method of Claim 1, wherein said mass spectrometry apparatus
comprises an ESI oa TOF mass spectrometry apparatus.

9.   The method of Claim 1, further comprising the step of d) displaying at
10   least said first physical property of at least a portion of said second separated protein
sample.

10.   The method of Claim 9, wherein said displaying comprises a schematic
representation of first and second physical properties of at least a portion of said
second separated protein sample.

15   11.   The method of Claim 10, wherein said first and second properties
comprise pI and hydrophobicity.

12.   The method of Claim 10, wherein said schematic representation
comprises a 2-dimensional protein map, wherein said first physical property is
represented on a first axis and wherein said second physical property is represented on
20   a second axis.

13.   The method of Claim 10, wherein said schematic representation further
displays protein abundance of proteins represented in said schematic representation.

14. The method of Claim 13, wherein proteins are represented as bands in said schematic representation.

15. The method of Claim 14, wherein protein abundance correlates to intensity of said bands.

5 16. The method of Claim 14, wherein said schematic representation has a resolution that allows the differentiation of a first band representing a first protein and a second band representing a phosphorylated version of said first protein.

17. The method of Claim 1, wherein said sample comprising a plurality of proteins further comprises a buffer, wherein said plurality of proteins are solubilized in
10 said buffer and wherein said buffer is compatible with said first and said second separating apparatus.

18. The method of Claim 17, wherein said buffer is further compatible with said mass spectrometry apparatus.

19. The method of Claim 17, wherein said buffer comprises a compound of
15 the formula n-octyl C_6-C_{12} glycopyranoside.

20. The method of Claim 19, wherein said compound of the formula n-octyl C_6-C_{12} glycopyranoside is selected from n-octyl ß-D-glucopyranoside and n-octyl ß-D-galactopyranoside.

21. A system comprising:
20 a) a first separating apparatus that separates proteins based on a first physical property; and

b) a non-porous reverse phase HPLC apparatus directly connected to a mass spectrometry apparatus, wherein proteins separated by said non-

porous reverse phase HPLC apparatus are directly fed to said mass
spectrometry apparatus without intervening processing.

22.   The system of Claim 21, wherein said first separating apparatus
comprises a liquid phase separating apparatus.

5   22.   The system of Claim 22, wherein said liquid phase first separating
apparatus comprises an isoelectric focusing apparatus.

23. · The system of Claim 21, wherein said mass spectrometry apparatus
comprises an ESI oa TOF mass spectrometry apparatus.

24.   The system of Claim 21, further comprising a detector that detects
10   proteins separated by said second separating apparatus.

25.   The system of Claim 24, further comprising a processor configured to
run protein display software, wherein said protein display software produces a data
representation of detected proteins.

26.   The system of Claim 25, further comprising a display that displays said
15   data representation, wherein said first physical property, said second physical
properties, and protein abundance of at least a portion of said plurality of proteins are
represented.

27.   The system of Claim 26, wherein said first and second properties
comprise pI and hydrophobicity.

20   28.   The system of Claim 26, wherein said data representation comprises a
2-dimensional protein map, wherein said first physical property is represented on a
first axis and wherein said second physical property is represented on a second axis.

29.   The system of Claim 26, wherein proteins are represented as bands in said data representation.

30.   The system of Claim 29, wherein protein abundance correlates to intensity of said bands.

5   31.   The system of Claim 29, wherein said data representation has a resolution that allows the differentiation of a first band representing a first protein and a second band representing a phosphorylated version of said first protein.

32.   A method for characterizing proteins comprising:

    a)   providing:

10   i)   a sample comprising a plurality of proteins;

        ii)   a first separating apparatus;

        iii) a non-porous reverse phase HPLC apparatus; and

        iv)   a mass spectrometry apparatus;

    b)   treating said sample with said first separating apparatus to

15   produce a first separated protein sample, wherein said first separated protein sample is collected from said first separating apparatus in a plurality of fractions, each of said fractions defined by a distinct pH range;

    c)   treating at least a portion of said first separated protein sample from at least one of said fractions with said non-porous reverse phase HPLC

20   apparatus to produce a second separated protein sample; and

    d)   mass spectrally analyzing at least a portion of said second separated protein sample with said mass spectrometry apparatus to characterize protein mass.

33.   The method of Claim 32, wherein said second separated protein sample

25   is directly fed to said mass spectrometry apparatus without an intervening protein digestion step.

34.   The method of Claim 32, wherein said sample comprises a cell lysate.

35.   The method of Claim 32, wherein said first separating apparatus separates proteins base on charge.

36.   The method of Claim 35, wherein said first separating apparatus
5   comprises an isoelectric focusing apparatus.

37.   The method of Claim 32, wherein said first separating apparatus comprises a liquid phase separating apparatus.

38.   The method of Claim 32, wherein said mass spectrometry apparatus comprises an ESI oa TOF mass spectrometry apparatus.

10   39.   The method of Claim 32, further comprising the step of e) displaying at least a first physical property of at least a portion of said second separated protein sample.

40.   The method of Claim 39, wherein said displaying comprises a schematic representation of first and second physical properties of at least a portion of said
15   second separated protein sample.

41.   The method of Claim 40, wherein said first and second properties comprise pI and hydrophobicity.

42.   The method of Claim 40, wherein said schematic representation comprises a 2-dimensional protein map, wherein said first physical property is
20   represented on a first axis and wherein said second physical property is represented on a second axis.

43.   The method of Claim 40, wherein said schematic representation further displays protein abundance of proteins represented in said schematic representation.

44.   The method of Claim 32, wherein said sample comprising a plurality of proteins further comprises a buffer, wherein said plurality of proteins are solubilized in said buffer and wherein said buffer is compatible with said first and said second separating apparatus.

45.   The method of Claim 44, wherein said buffer is further compatible with said mass spectrometry apparatus.

46.   The method of Claim 44, wherein said buffer comprises a compound of the formula n-octyl C_6-C_{12} glycopyranoside.

47.   The method of Claim 46, wherein said compound of the formula n-octyl C_6-C_{12} glycopyranoside is selected from n-octyl ß-D-glucopyranoside and n-octyl ß-D-galactopyranoside.

48.   A system comprising:
       a)   a first separating apparatus;
       b)   a buffer that elutes protein from said first separating apparatus in a plurality of fractions, each of said fractions defined by a distinct pH range;
       c)   a first delivery apparatus capable of receiving separated protein in said plurality of fractions;
       d)   a non-porous reverse phase HPLC apparatus configured to receive proteins from said first delivery apparatus; and
       e)   a mass spectrometry apparatus configured to receive proteins from said non-porous reverse phase HPLC apparatus.

49.   The system of Claim 48, wherein said mass spectrometry apparatus is configured to directly receive proteins from said non-porous reverse phase HPLC apparatus.

50.   The system of Claim 48, wherein said first separating apparatus

5   comprises a liquid phase separating apparatus.

51.   The system of Claim 50, wherein said liquid phase first separating apparatus comprises an isoelectric focusing apparatus.

52.   The system of Claim 48, wherein said mass spectrometry apparatus comprises an ESI oa TOF mass spectrometry apparatus.

10   53.   The system of Claim 48, further comprising a detector that detects proteins separated by said non-porous reverse phase HPLC apparatus.

54,   The system of Claim 53, further comprising a processor configured to run protein display software, wherein said protein display software produces a data representation of detected proteins.

15   55.   The system of Claim 54, further comprising a display that displays said data representation, wherein a first physical property, a second physical properties, and protein abundance of at least a portion of said plurality of proteins are represented.

56.   The system of Claim 55, wherein said first and second properties comprise pI and hydrophobicity.

20   57.   The system of Claim 55, wherein said data representation comprises a 2-dimensional protein map, wherein said first physical property is represented on a first axis and wherein said second physical property is represented on a second axis.

58.   An automated protein characterization system comprising:

a)   a first separating apparatus;

b)   an automated sample handling device;

c)   a second separating apparatus operably linked to said first separating apparatus and said sample handling device, wherein said second separating apparatus is configured to receive proteins from said first separating apparatus;

d)   a mass spectrometry apparatus operably linked to said second separating apparatus and said sample handling device; wherein said mass spectroscopy apparatus is configured to receive proteins from said second separating apparatus; and

e)   a processor that controls said sample handling device, said first separating apparatus, said second separating apparatus; and said mass spectrometry apparatus.

59.   The system of Claim 58, wherein said processor comprises computer memory and a computer processor.

60.   The system of Claim 58, wherein said processor is configured to produce a data representation of separated proteins analyzed by said mass spectrometry apparatus.

61.   The system of Claim 58, wherein said automated sample handling device comprises a switchable, multi-channel valve.

62.   The system of Claim 60, further comprising a display that displays said data representation.

63.   The system of Claim 60, wherein said data representation comprises a first dimension representing protein charge.

64.   The system of Claim 63, wherein said representation further comprises a second dimension representing protein molecular weight.

65.   The system of Claim 65, wherein said data representation further represents protein abundance.

5   66.   The system of Claim 58, wherein said first separating apparatus comprises a liquid phase separating apparatus.

67.   The system of Claim 66, wherein said liquid phase first separating apparatus comprises an isoelectric focusing apparatus.

68.   The system of Claim 58, wherein said second separating apparatus
10   comprises a non-porous reverse phase HPLC apparatus.

69.   The system of Claim 58, wherein said mass spectrometry apparatus comprises an ESI oa TOF mass spectrometry apparatus.

70.   The system of Claim 68, wherein said mass spectrometry apparatus is configured to directly receive proteins from said non-porous reverse phase HPLC
15   apparatus.

71.   The system of Claim 58, further comprising a solid phase extraction apparatus configured to treat proteins separated by said first separating apparatus prior to delivery of proteins to said second separating apparatus.

72.   An automated method for separating proteins comprising:
20   a)   providing:
                   i)   a sample comprising a plurality of proteins,

ii)   a first separating apparatus that separates proteins based on a first physical property;

iii)   a second separating apparatus that separates proteins based on a second physical property;

5   iv)   a mass spectroscopy apparatus; and

v)   an automated sample handling device comprising a switchable, multi-channel valve;

b)   treating said sample with said first separating apparatus to produce a first separated protein sample, wherein said first separated protein

10   sample is collected from said first separating apparatus in a plurality of fractions, each of said fractions defined by a distinct range of said first physical property;

c)   transferring said first separated protein sample to said second separating apparatus using said automated sample handling device;

15   d)   treating said first separated protein sample with said second separating apparatus to produce a second separated protein sample;

e)   transferring said second separated protein sample to said mass spectroscopy apparatus using said automated sample handling device; and

f)   mass spectrally analyzing said second separated protein sample

20   with said mass spectroscopy apparatus to characterize protein mass.

73.   The method of Claim 72, further comprising a centralized control network operably linked to said automated sample handling device, said first separating apparatus, said second separating apparatus, and said mass spectroscopy apparatus, wherein said centralized control network is configured to control said

25   automated sample handling device, said first separating apparatus, said second separating apparatus, and said mass spectroscopy apparatus.

74.   The method of Claim 72, further comprising providing a solid phase extraction apparatus, wherein prior to treating said first separated sample with said

second apparatus; said first separated sample is treated with said solid phase extraction apparatus.

75.   The method of Claim 72, wherein said sample comprises a cell lysate.

76.   The method of Claim 72, wherein said first physical property is protein

5   charge.

77.   The method of Claim 72, wherein said first separating apparatus comprises an isoelectric focusing apparatus.

78.   The method of Claim 72, wherein said first separating apparatus comprises a liquid phase separating apparatus.

10   79.   The method of Claim 72, wherein said second separating apparatus comprises a reverse phase HPLC apparatus.

80.   The method of Claim 79, wherein said reverse phase HPLC comprises non-porous reverse phase HPLC.

81.   The method of Claim 72, wherein said mass spectrometry apparatus

15   comprises an ESI oa TOF mass spectrometry apparatus.

82.   The method of Claim 72, further comprising the step of g) displaying at least said first physical property of at least a portion of said second separated protein sample.

83.   The method of Claim 82, wherein said displaying comprises a schematic

20   representation of first and second physical properties of at least a portion of said second separated protein sample.

84.   The method of Claim 83, wherein said first and second properties comprise pI and hydrophobicity.

85.   The method of Claim 83, wherein said schematic representation comprises a 2-dimensional protein map, wherein said first physical property is

5   represented on a first axis and wherein said second physical property is represented on a second axis.

86.   The method of Claim 83, wherein said schematic representation further displays protein abundance of proteins represented in said schematic representation.

87.   The method of Claim 72, wherein said sample comprising a plurality of

10   proteins further comprises a buffer, wherein said plurality of proteins are solubilized in said buffer and wherein said buffer is compatible with said first and said second separating apparatus.

88.   The method of Claim 87, wherein said buffer is further compatible with said mass spectrometry apparatus.

15   89.   The method of Claim 87, wherein said buffer comprises a compound of the formula n-octyl C_6-C_{12} glycopyranoside.

90.   The method of Claim 89, wherein said compound of the formula n-octyl C_6-C_{12} glycopyranoside is selected from n-octyl ß-D-glucopyranoside and n-octyl ß-D-galactopyranoside.

FIGURE 1
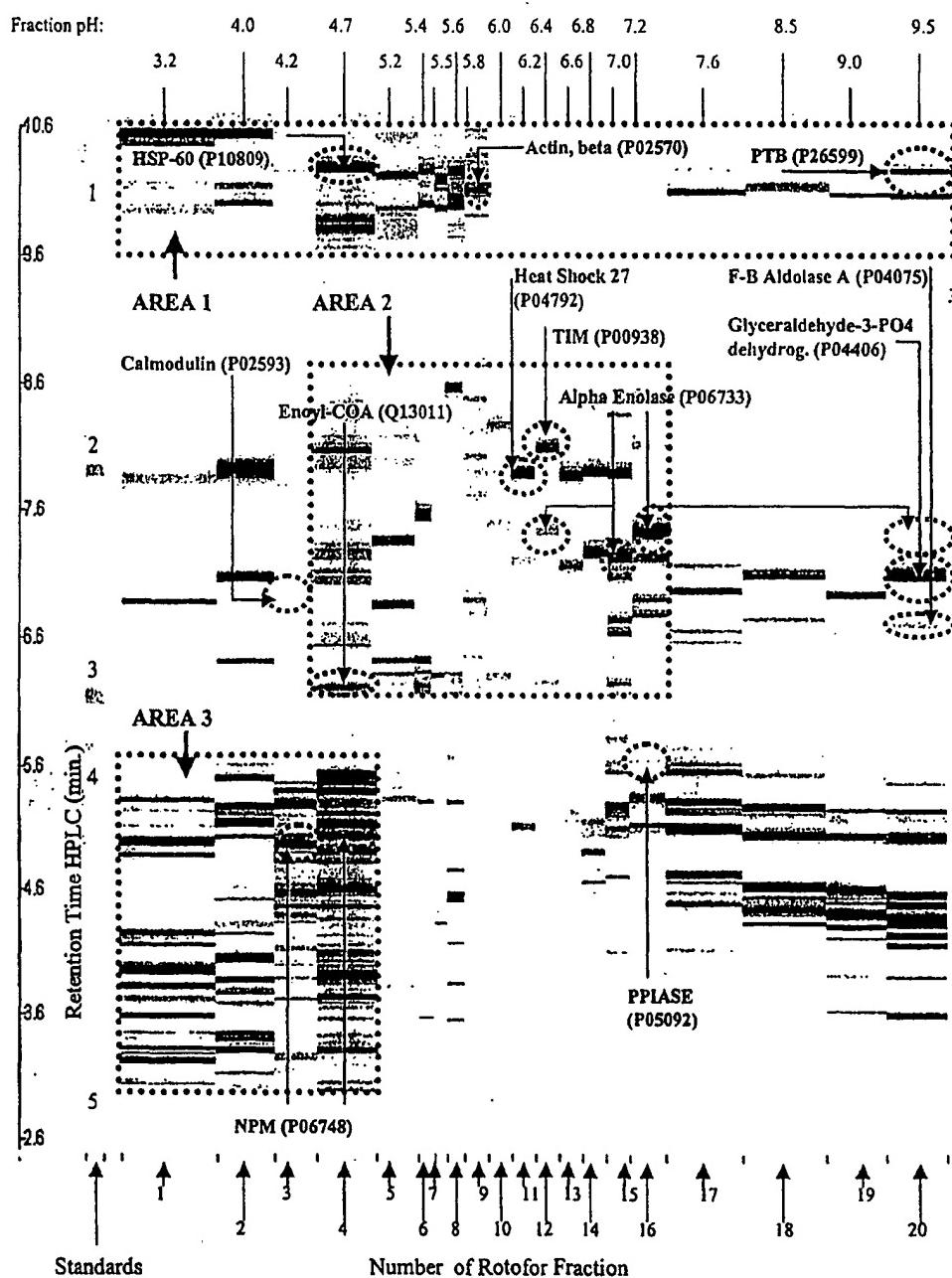
*1 = Ovalbumin (45 kDa), 2 = Carbonic Anhydrase (29 kDa), 3 = BSA (67 kDa),

FIGURE 2

FIGURE 3

Total Protein Load = 23.493 mg
Total Protein Recovered = 10.160 mg
% Protein Recovery = 43.247 %

FIGURE 4

FIGURE 5

A — W = 0.14 and tG. = 10.5 so peak capacity = 10.5/0.14 = 75
actual number of peaks = 35

B — W = 0.23 and tG = 21 so peak capacity = 21/0.23 = 91
actual number of peaks = 51
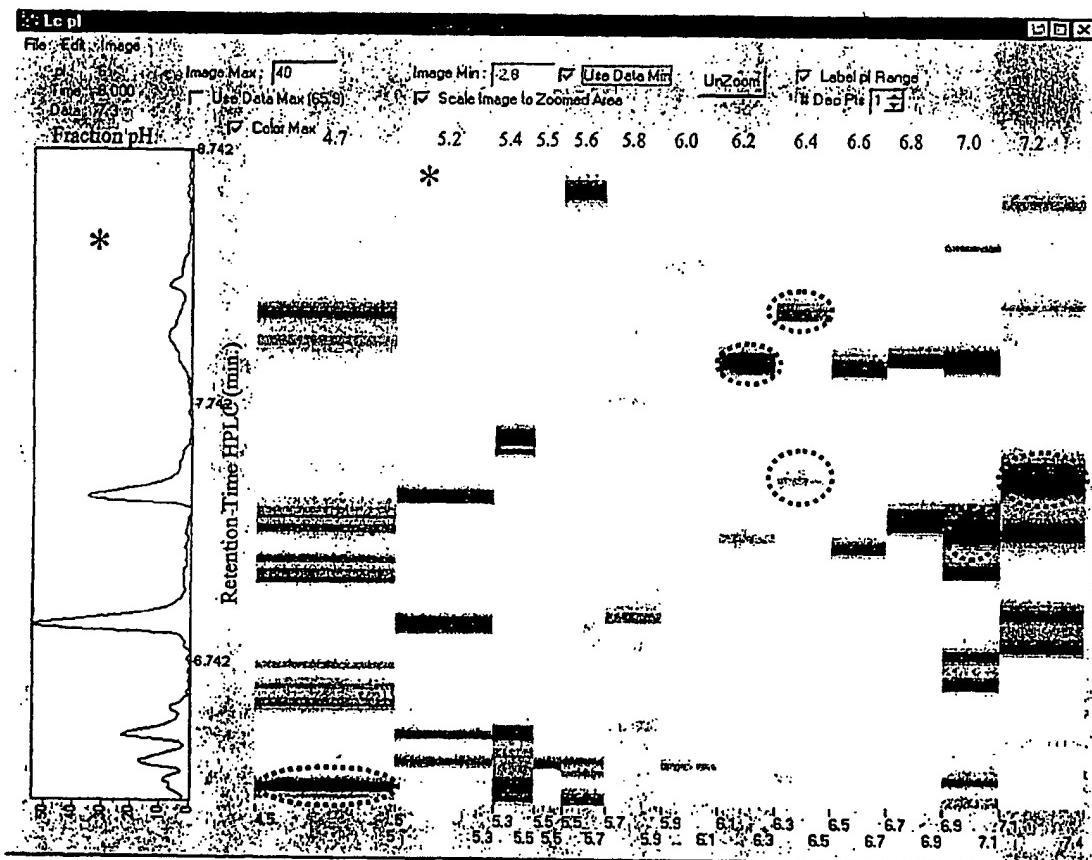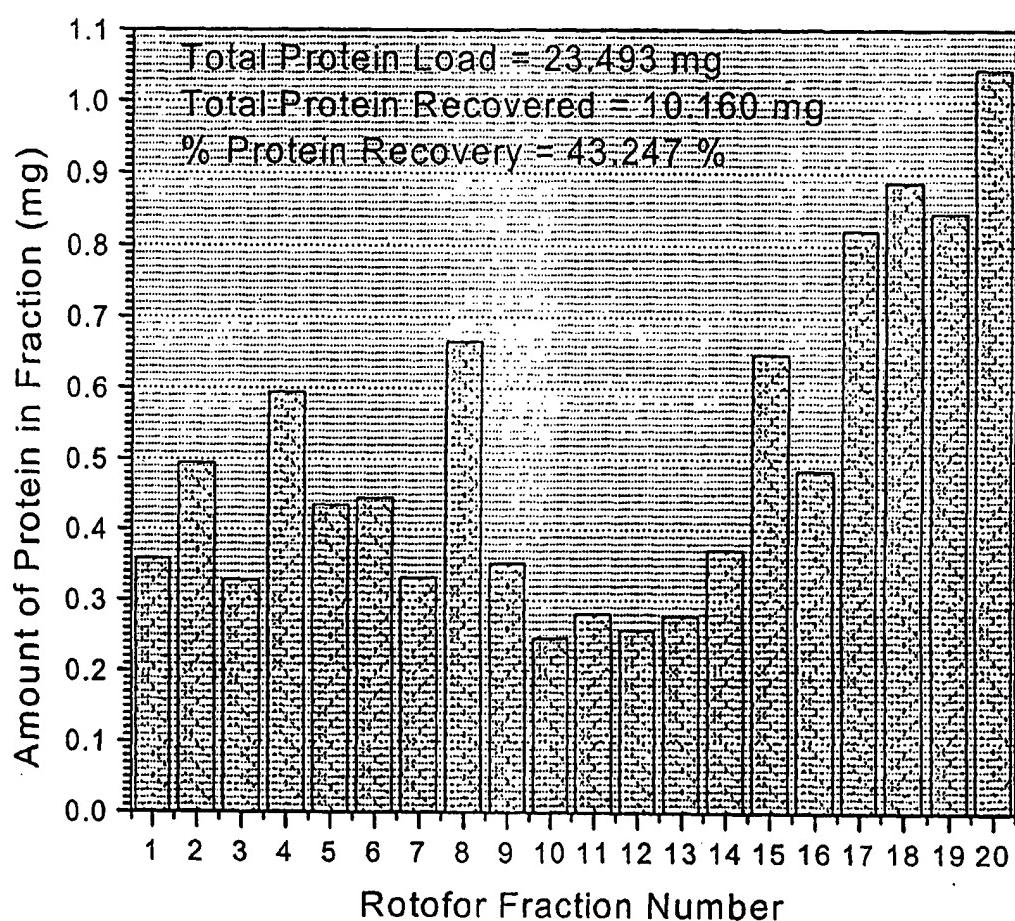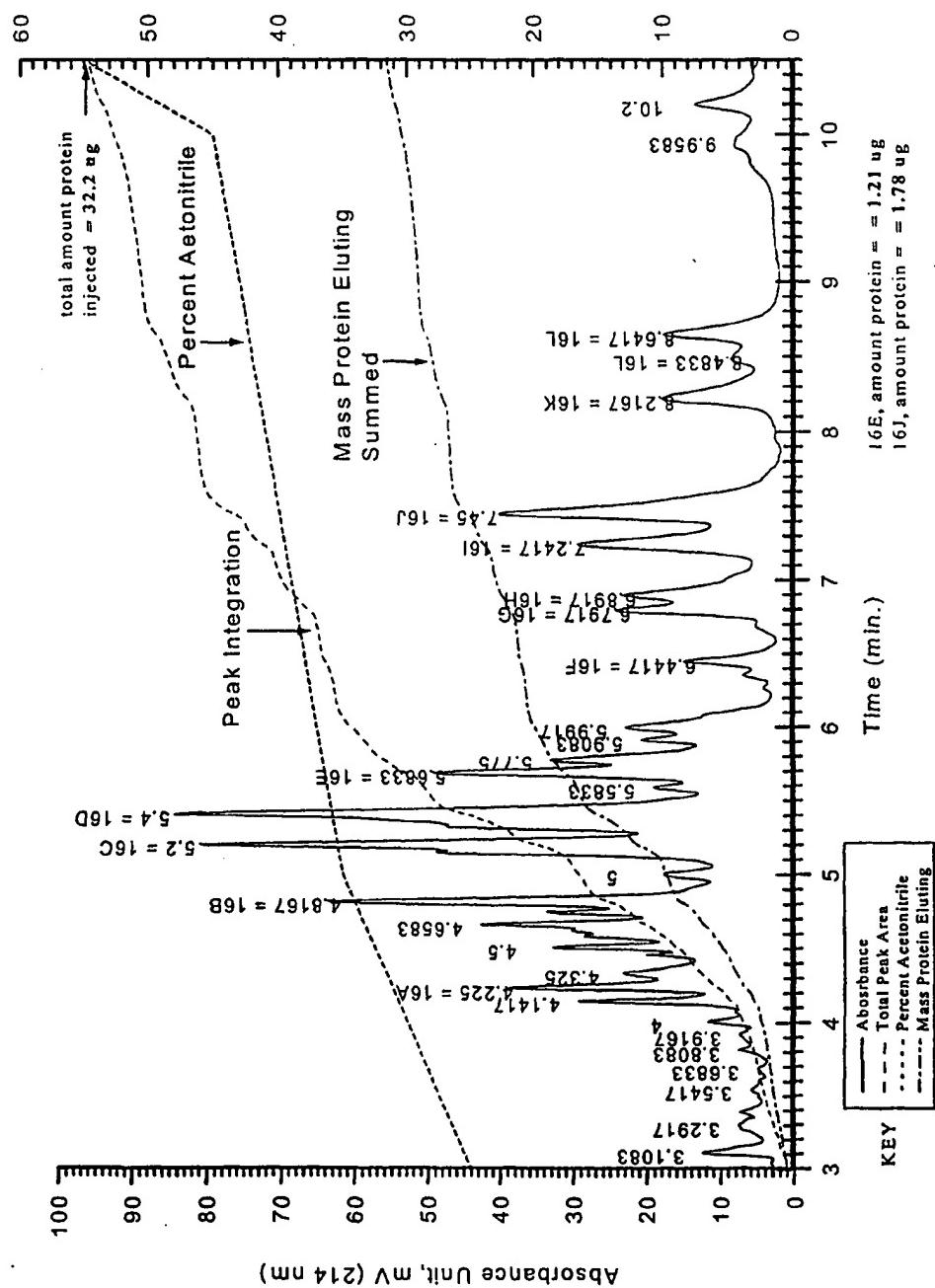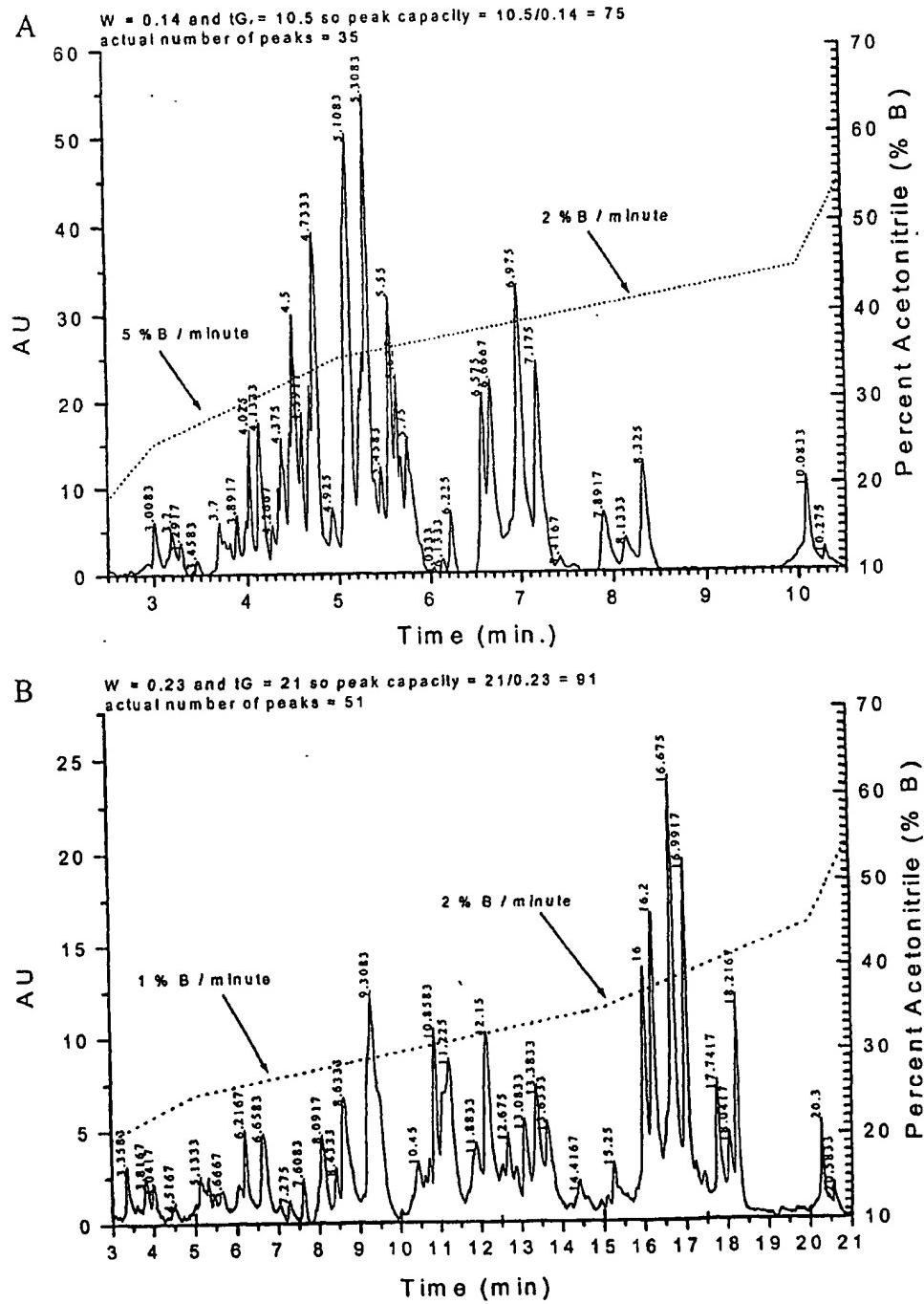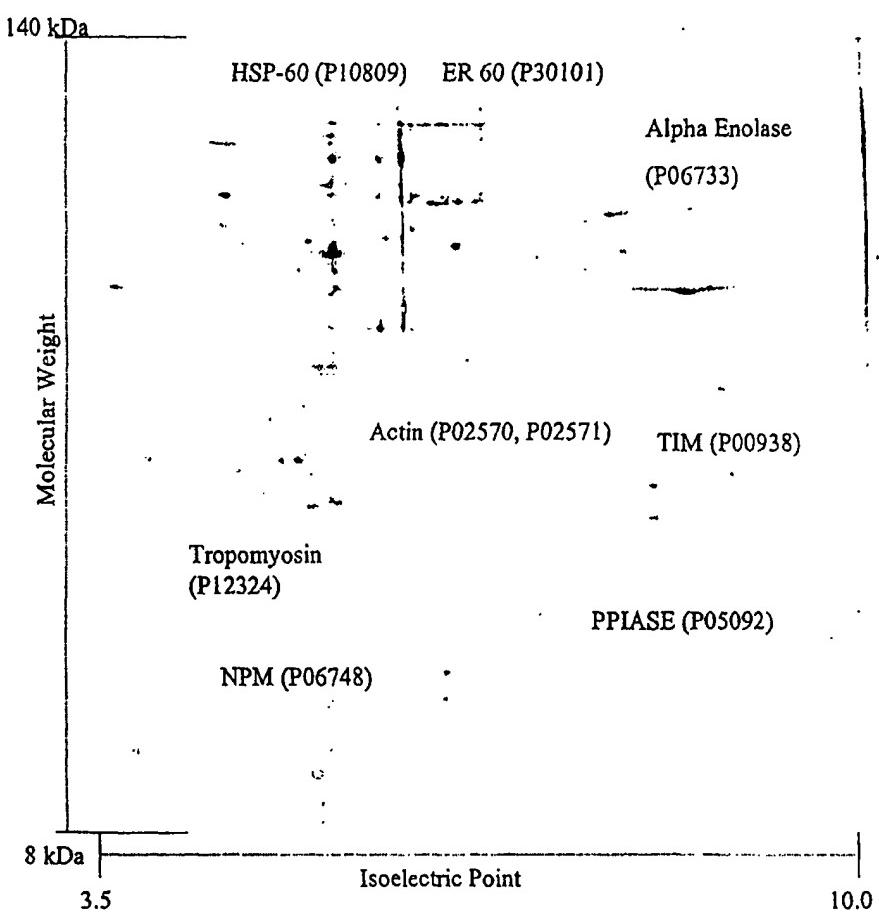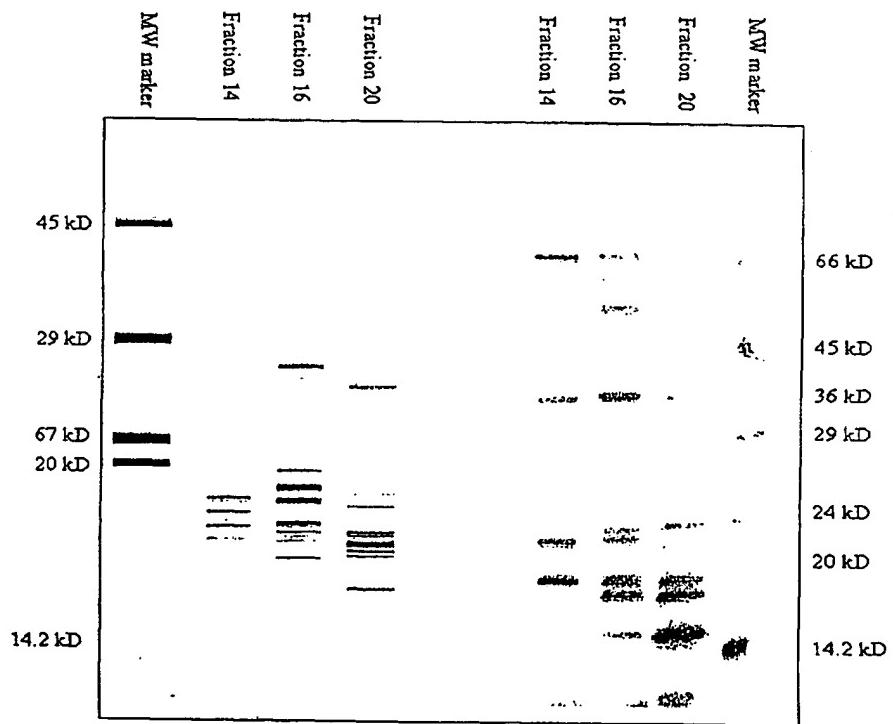
FIGURE 6

FIGURE 7

FIGURE 8A
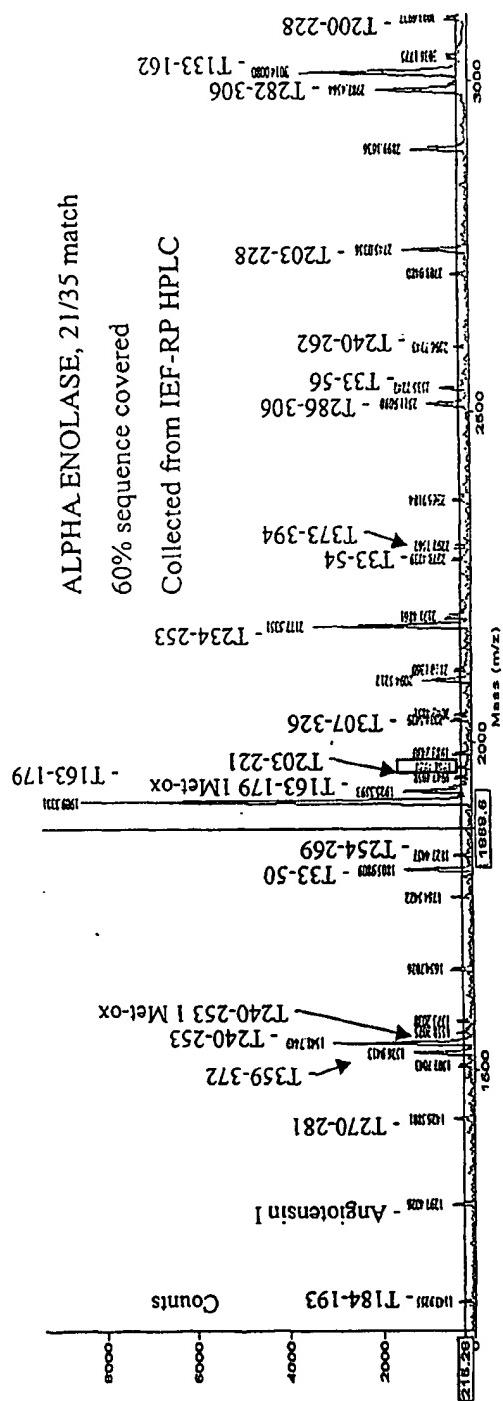
FIGURE 8B
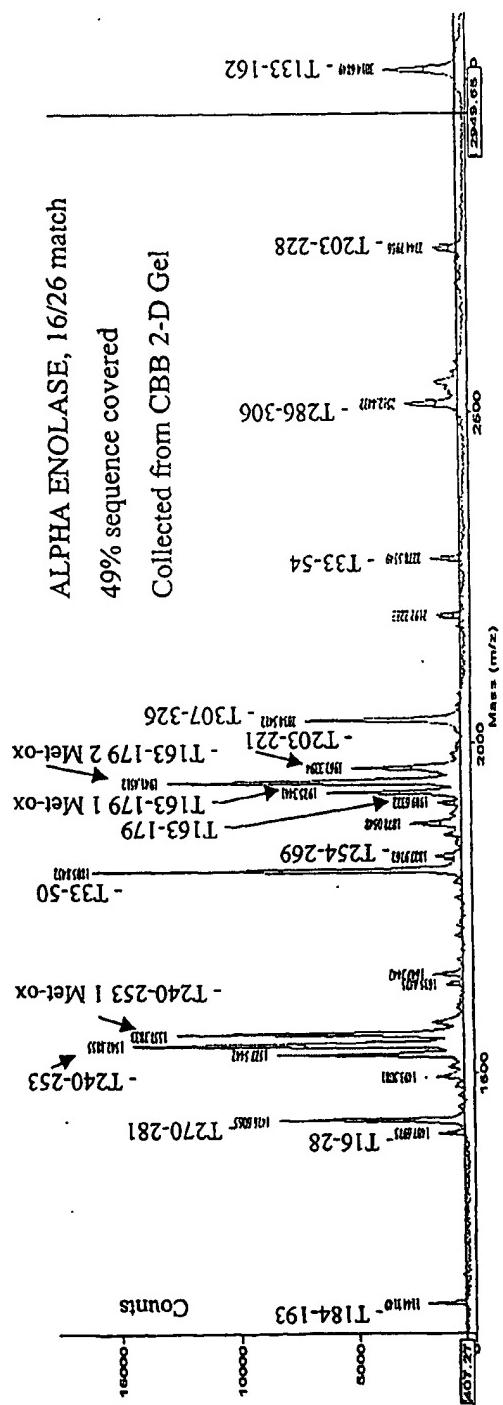
FIGURE 9
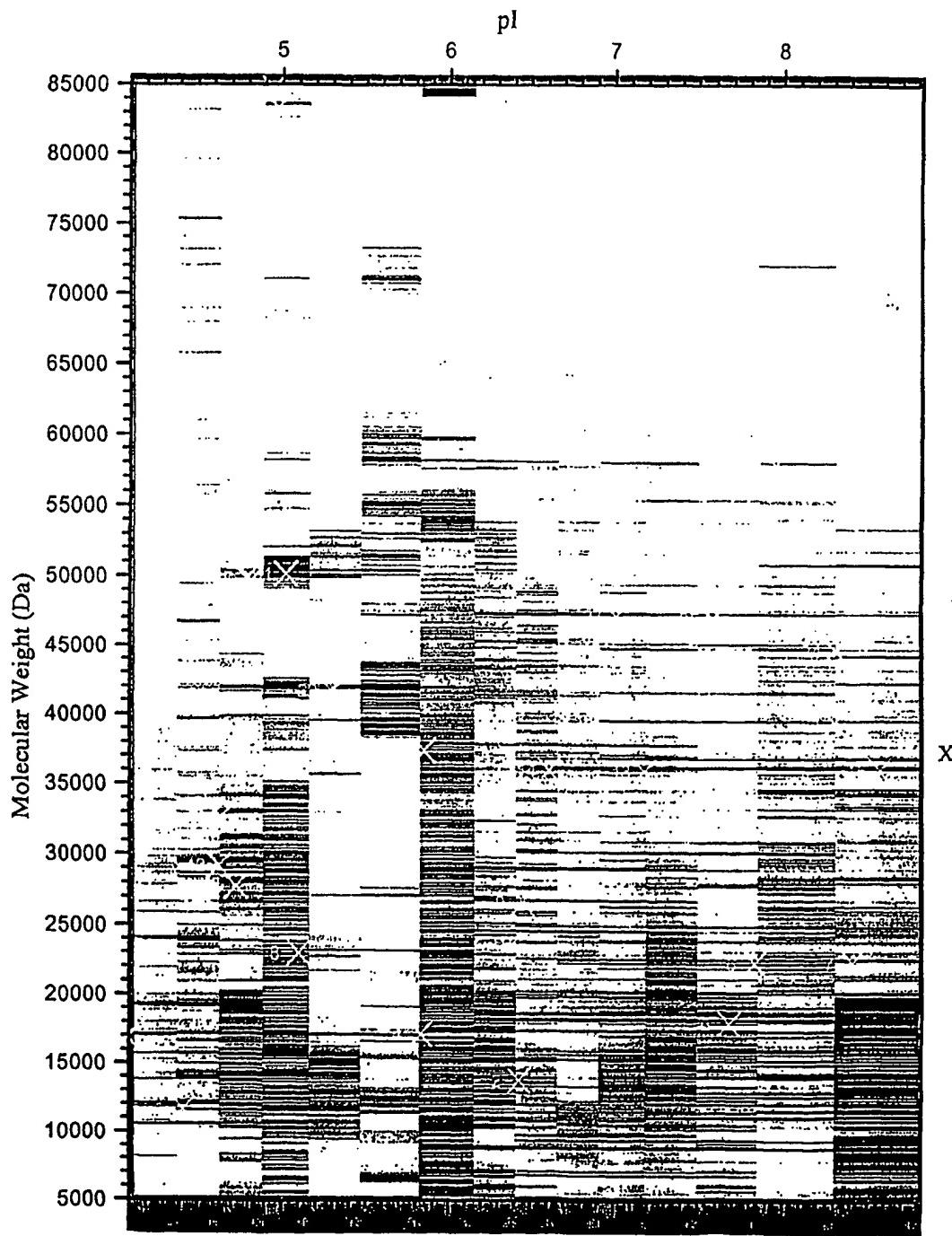
Key: the X marks the database coordinates of a given protein

FIGURE 10

FIGURE 11

MaxEnt Values Actin
Beta: 41662.8 Da
Gamma: 41720.6 Da
Gamma-Beta: 57.8 Da

Database Values Actin
Beta: 41737.0
Gamma: 41793.1
Gamma-Beta: 56.1 Da
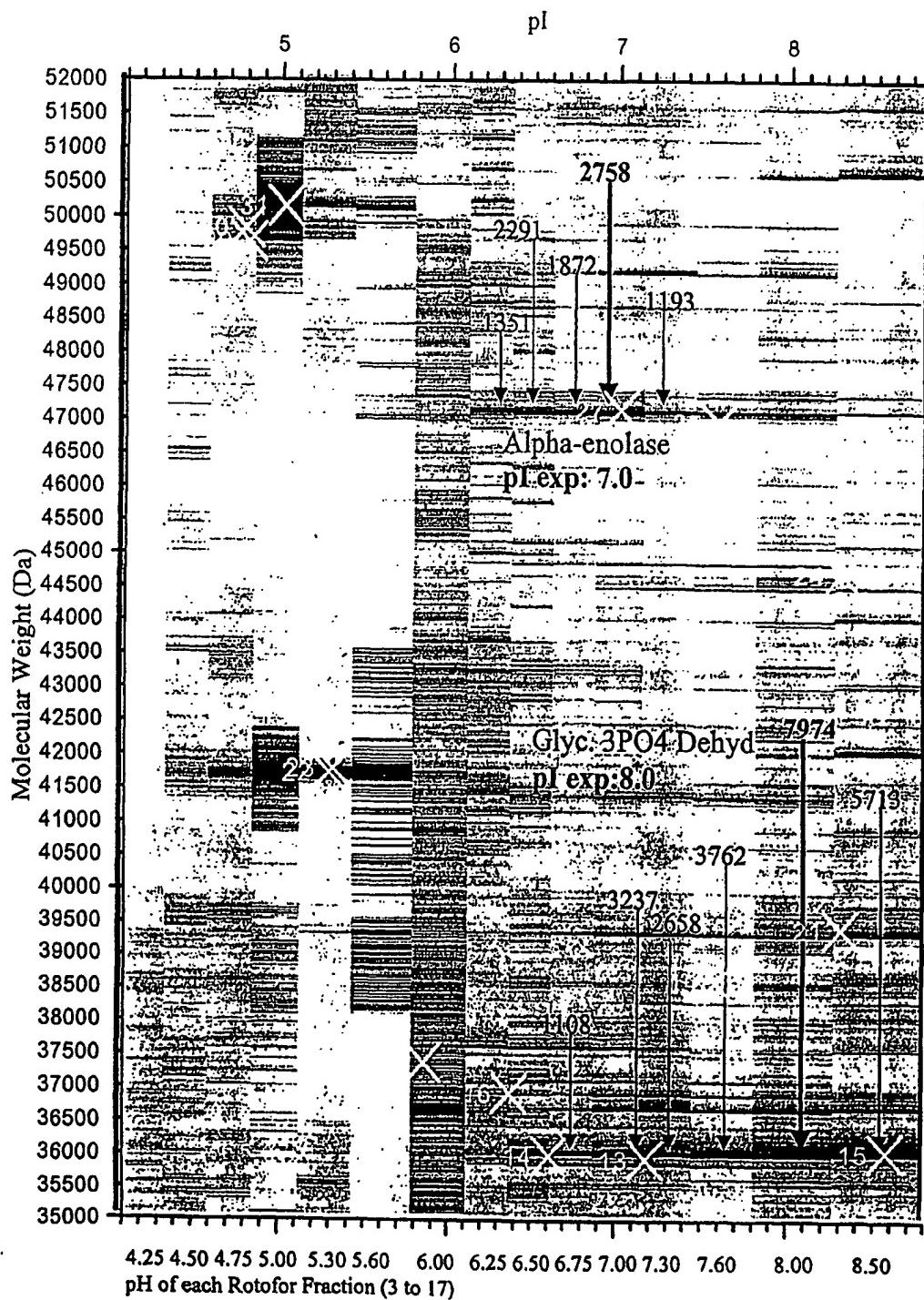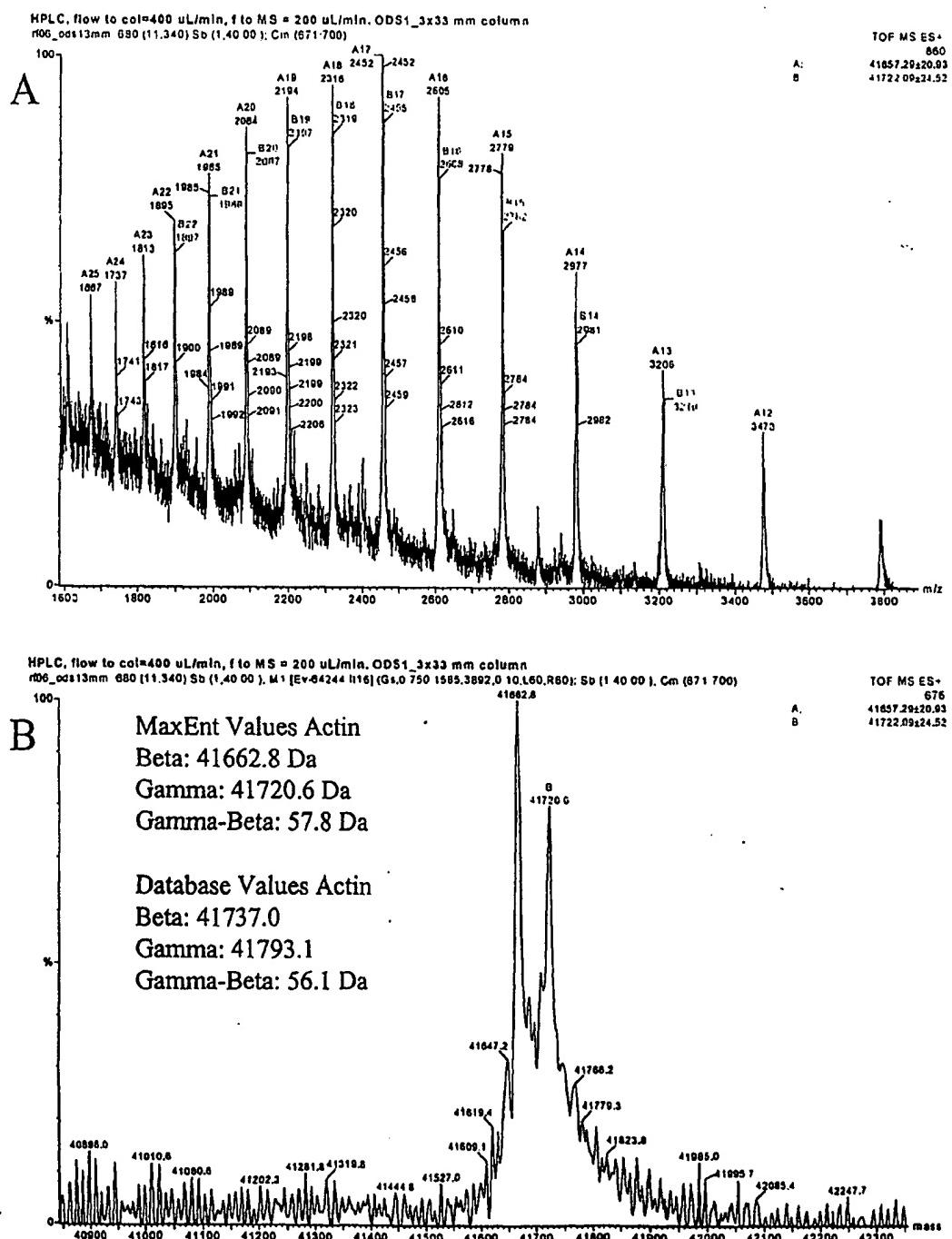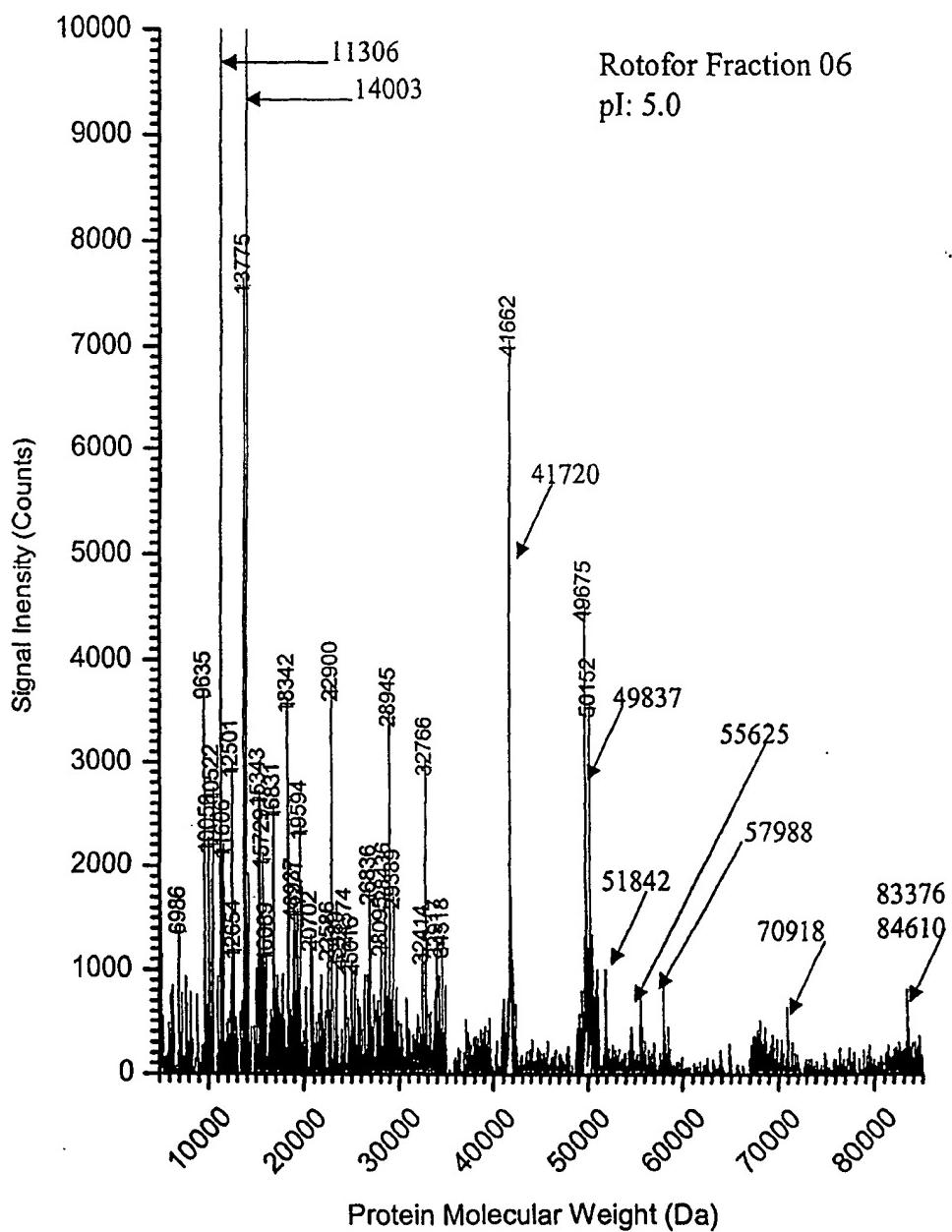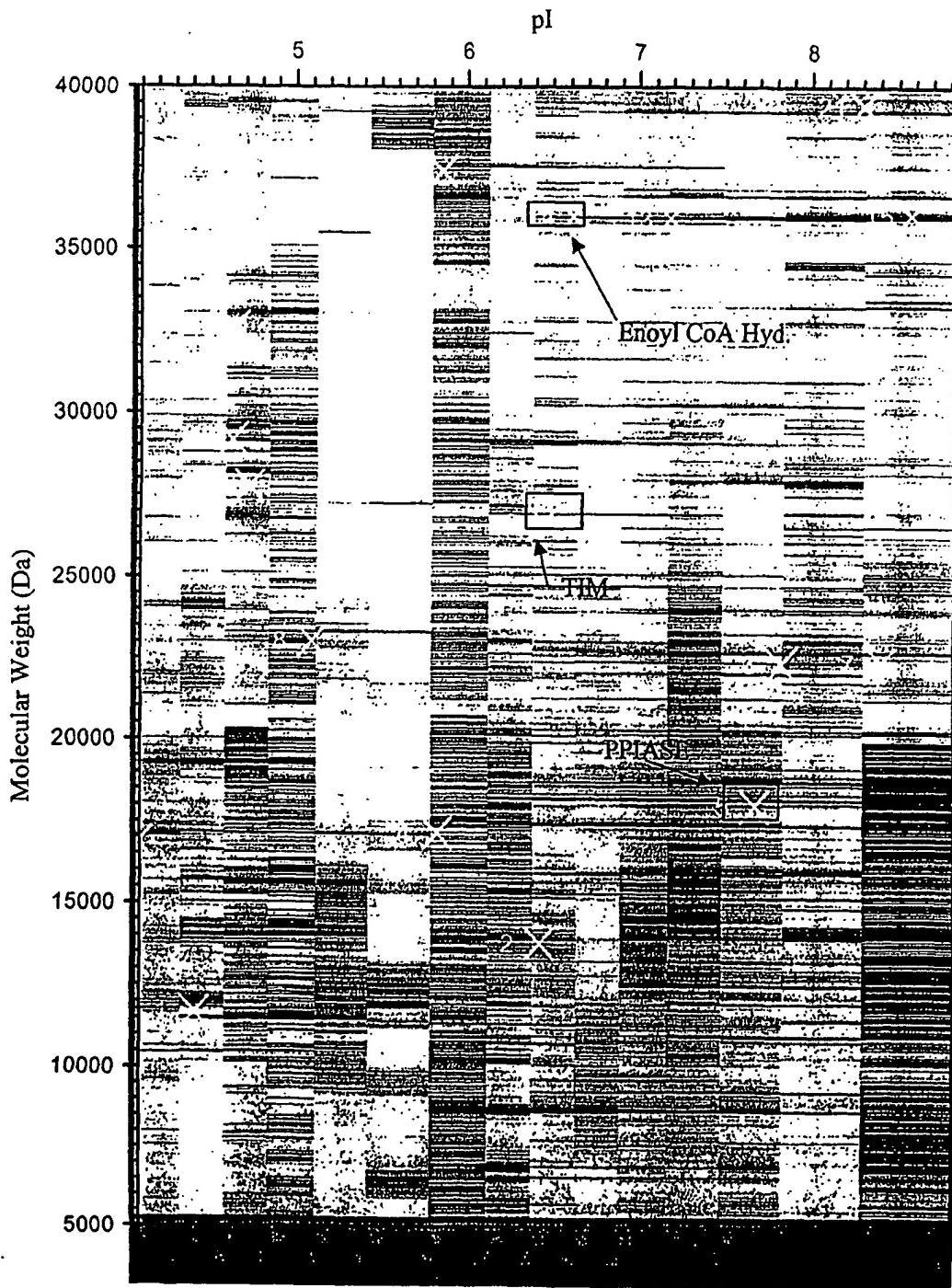
FIGURE 12

FIGURE 13

FIGURE 14
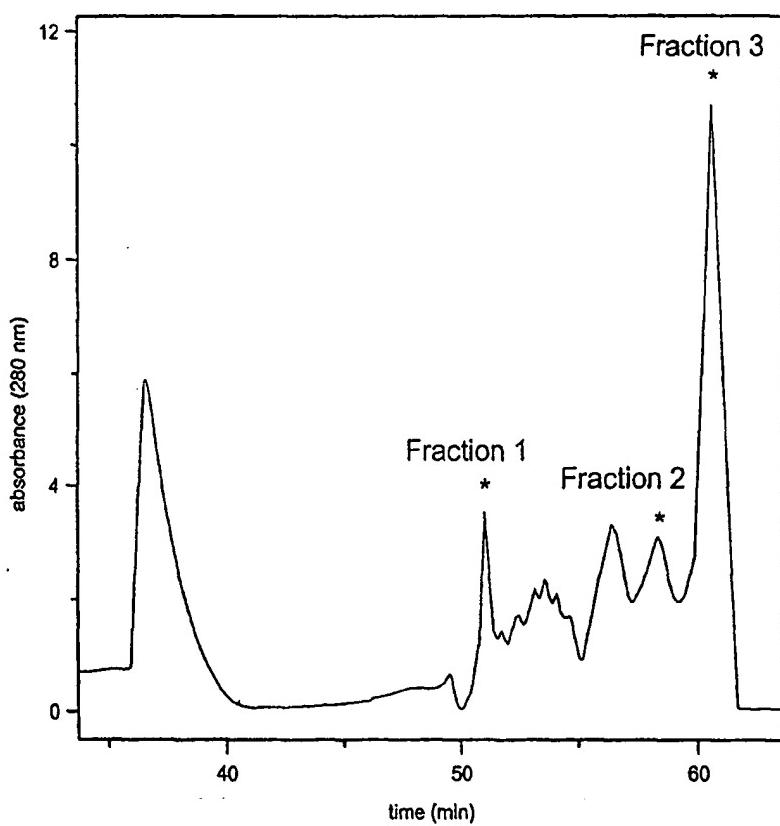
FIGURE 15

A Fraction 1 (pH 6.75 – 6.55)
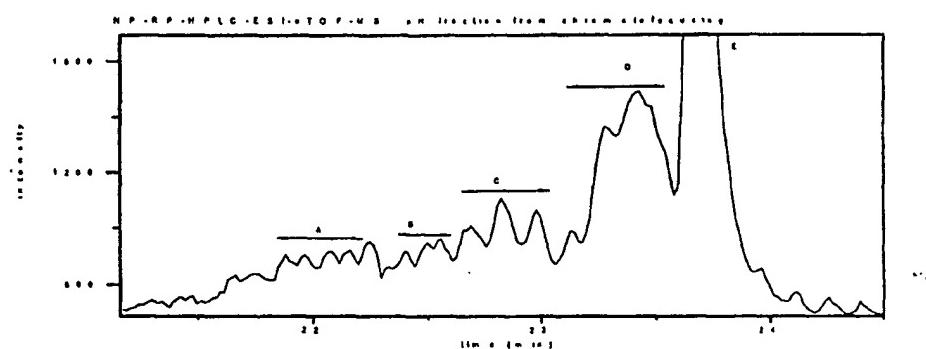
B Fraction 2 (pH 5.50 – 5.25)
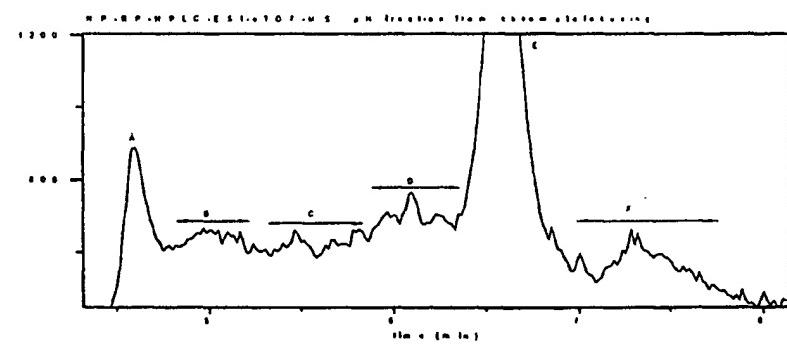
C Fraction 3 (pH 5.20 – 4.90)
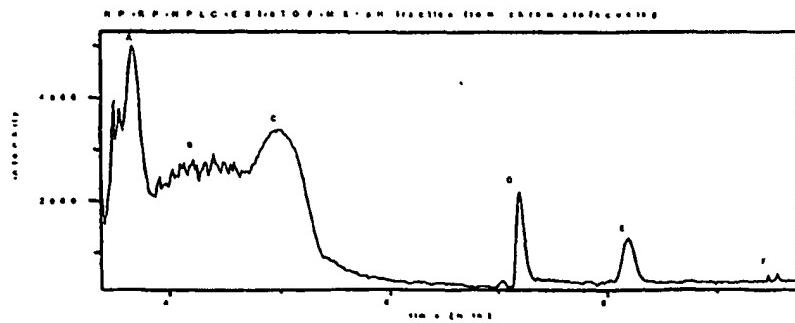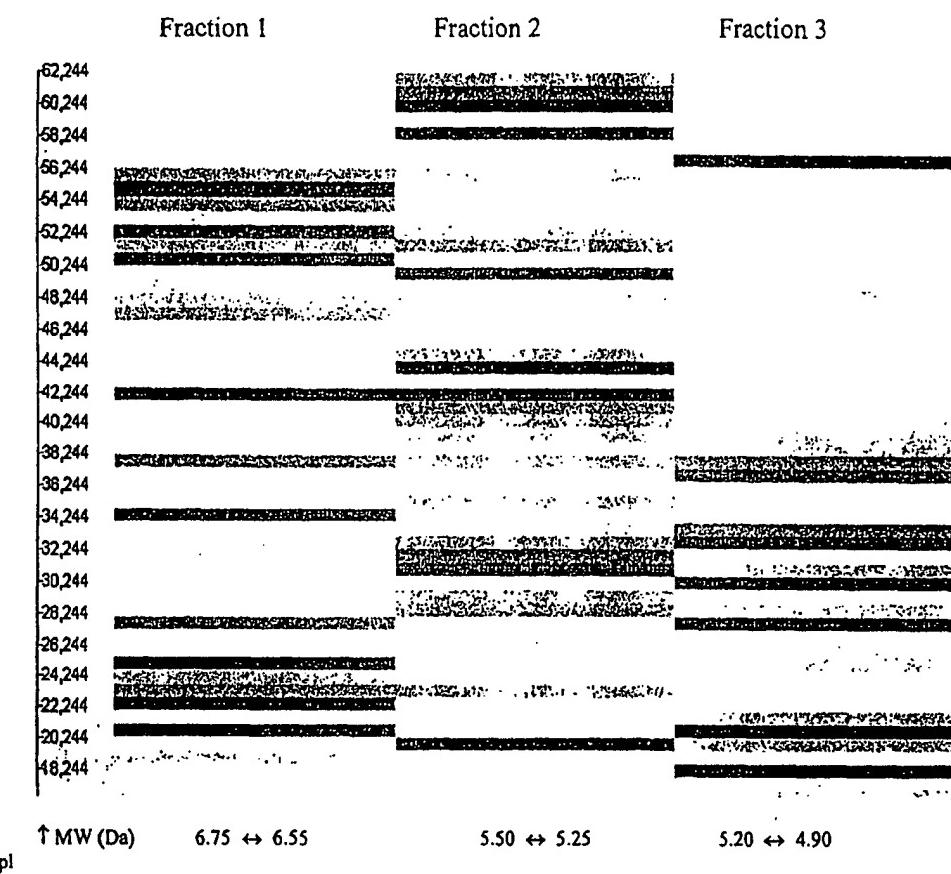
FIGURE 16

Figure 17
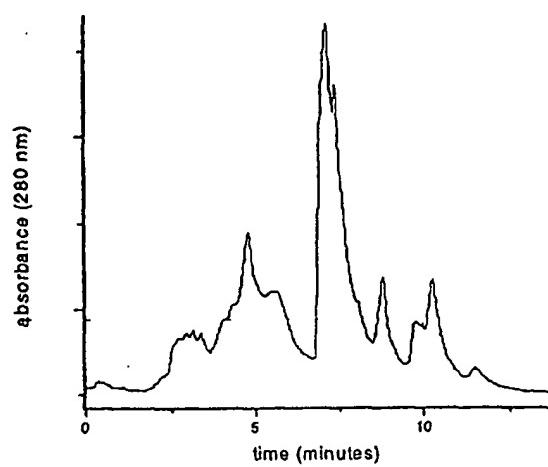
Figure 18

(a) Fraction collected from 3 to 4 minutes

(b) Fraction collected from 7 to 8 minutes